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# CLINICAL CHEMISTRY

*Journal of the American Association of Clinical Chemists*


". . . a forum where chemists engaged in applying the science of clinical chemistry may exchange ideas and information concerning their scientific, technical and professional problems."


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# Diagnostic Biochemistry and Clinical Medicine: Facts and Fallacies

*Oscar Bodansky*

AT AN EVER-INCREASING TEMPO within the past 50 years, biochemistry has been contributing greatly to clinical medicine. These contributions have not only elucidated the mechanisms of various diseases but have also provided practical procedures for their diagnosis and the management of the individual patient. Some of the contributions that have taken place during the past decade will be noted in the first part of this paper. Although the clinician is generally appreciative of these contributions, he occasionally has reservations concerning the specific results that he obtains from the diagnostic biochemistry laboratory. The second part of this paper will be devoted to a consideration of the bases for these reservations. In the third section, we shall attempt to describe the type of cooperation and understanding that should exist between the clinician and the clinical biochemist.

## Recent Advances in Clinical Applications of Biochemistry

The development of easily executed, quantitative methods for the determination of blood and urinary biochemical components occurred during the first and second decades of the present century. Venipuncture became an accepted procedure, and the second and third decades were characterized by the development of the relationships between

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various pathologic processes and alterations in the concentration of blood chemical components. Diabetes and diseases of the liver and of the kidney were of particular interest in this connection.

Tables 1 and 2 show the mortality rates and, in certain instances in which information is reportable, the prevalence or incidence of the major diseases in the United States. In some of these diseases, biochemistry played an important role as an aid in diagnosis and management. Because of the inevitable extension of medical knowledge and medical care, the clinician has become increasingly interested in the rarer diseases. In many of these diseases, particularly those having a hereditary basis, diagnostic biochemistry has begun to play a distinctive role.

Two major areas of advance in diagnostic biochemistry have been discussed in detail by other contributors to this symposium. Here I should like to note briefly several other areas that have received attention during the past few years.

## Clinical Enzymology

The diagnostic use of the serum alkaline and acid phosphatases, amylase, and lipase has been widely accepted for about 25–30 years.

**Table 1.** Prevalence of and Mortality in Various Diseases in the United States

| *Disease* | *Prevalence** | *Mortality†* |
|---|---|---|
| Malignant neoplasms | 430 | 147.4 |
| Diabetes mellitus | 1500 | 17.1 |
| Tuberculosis, all forms | 150 | 5.9 |
| Cirrhosis of liver | | 11.2 |
| Diseases of heart | | 366.4 |
| Anemias | | 1.9 |
| Congenital malformations | | 12.0 |

*Prevalence data (per 100,000) based on Cecil and Loeb (*30*).
†Death rates, 1960 (per 100,000) from the *World Almanac* (*31*).

**Table 2.** Incidence of and Mortality in Infectious Diseases in the United States*

| *Disease* | *Incidence* | *Mortality* |
|---|---|---|
| Influenza | 80.0 | 2.55 |
| Infectious hepatitis | 9.3 | 0.51 |
| Rheumatic fever | 3.8 | 0.46 |
| Acute poliomyelitis | 3.3 | 0.034 |
| Meningococcal infections | 1.5 | 0.43 |

*Calculated per 100,000 on basis of total population of 175,000,000. Data from *Annual Epidemiological and Vital Statistics for 1958* (*32*).

These enzymes are in general characterized by their high concentrations in only a few tissues, and diseases of these tissues are therefore revealed by relatively specific elevations of enzyme activity in the serum. Enzymes that are involved in metabolic actions are likely to be present in substantial concentrations in almost all tissues, and in general, these enzymes are not too specific for the tissue damage or the disease causing the damage. However, the levels of these enzymes in serum may vary because of some differences in tissue distribution, size of organ, ease of passage of enzyme from the tissue, and the rate of disposal from the circulation (*1*). The main serum enzymes reported during the last decade to have diagnostic potentialities are the following (1): adenosine deaminase (1957), alcohol dehydrogenase (1958), glucose-6-phosphatase (1959), glucose-6-phosphate dehydrogenase (1958), glutamic oxaloacetic transaminase (1955), glutamic pyruvic transaminase (1956), glutathione reductase (1958), isocitric dehydrogenase (1958), lactic dehydrogenase (1954), leucine aminopeptidase (1954), malic acid dehydrogenase (1956), 5-nucleotidase (1958), ornithine transcarbamylase (1957) pepsin (1952), peptidases (1953, 1954), 6-phosphogluconic dehydrogenase (1957), phosphoglucomutase (1957), phosphohexose isomerase (1953), phosphoribose isomerase (1956), and ribonuclease (1958). There are two especially useful aspects of these enzymes: First, changes in their levels are often the first and only criterion of impending disease (*2*). Secondly, one or another of these enzymes, depending upon the particular disease, acts as a good and sometimes excellent guide to the progress, abatement, or recrudescence of the disease (*3, 4*).

**Table 3.** Serum Leucine Aminopeptidase in Hepatic and Other Diseases*

| *Condition* | *Values as percentage of normal mean* |
|---|---|
| Normal | 52-148† |
| Non-neoplastic; no hepatic or pancreatic involvement | 35-120 |
| Carcinoma of pancreas, no obstruction or metastases | 95-126 |
| Acute pancreatitis | 120-330 |
| Acute cholecystitis | 152-375 |
| Carcinoma of pancreas with obstruction or metastases | 210-660 |
| Intrahepatic metastases | 104-730 |
| Viral hepatitis | 120-460 |
| Hepatitis with intrahepatic cholestasis | 560-960 |

*Based on review of literature in Reference *2*.

†This is equivalent to mean ± 2 standard deviations. The actual units are 128 ± S.D. of 31 units.

Some of these enzymes, it has been proposed, are fairly specific indicators of various groups of diseases. Table 3 shows that leucine aminopeptidase is an indicator, not of pancreatic disease, as was originally proposed, but rather of hepatic involvement. Isocitric dehydrogenase (Table 4) and 5-nucleotidase (Table 5) are also indicators of hepatic diseases, but their concentrations do not differentiate among the various types.

The correlation of subcellular structure in human tissues with function at enzymatic levels is an interesting development in current investigations by biochemists and pathologists (*5*, *6*). A partial list of the association between subcellular structures in the liver and various enzyme or metabolic activities, based on the data of Novikoff and Essner (*5*) and of Schaffner and Popper (*6*), is as follows:

| *Structure* | *Associated enzymes or metabolic activity* |
|---|---|
| Nucleus | Synthesis of DPN |
| Mitochondria | Oxidative phosphorylation |
| Lysosome granules near bile canaliculi | Acid P'ase; cathepsin and other hydrolases |
| Bile canaliculi | 5-Nucleotidase; ATP'ase, alkaline P'ase |
| Microvilli on sinusoidal surface | 5-Nucleotidase; ATP'ase |
| Granular reticulum or ergastoplasm; ribosomes | Amino acid incorporation into protein |

Through the combined use of histochemistry, electron microscopy, and immunocytochemistry on material from biopsy and autopsy, Schaffner and Popper (*6*) have studied alterations in liver diseases. If this research proves fruitful, we may envision a situation in which the clinical biochemist will be called upon for enzymatic analyses of the various cellular structures from biopsy material.

An enzyme activity in the serum may represent a mixture of several catalytically similar enzymes from different tissues of the body or even

**Table 4.** Isocitric Dehydrogenase in Hepatic and Other Diseases*

| *Condition* | *Values as percentage of normal mean* |
|---|---|
| Normal | 0-200† |
| Myocardial infarct, recent | 77-250 |
| Muscular dystrophy | 89-260 |
| Leukemias | 156-540 |
| Intrahepatic metastases | 87-4100 |
| Viral hepatitis | 2180-7600 |

*Based on review of literature in Reference *3*.
†This is equivalent to mean ± 2 standard deviations.

**Table 5.** SERUM 5-NUCLEOTIDASE ACTIVITY IN HEPATIC AND BONE DISEASE*

| Condition | Mean (units) | Range (units) | % abnormal: Mildly elevated (1.7–5 U.) | % abnormal: Markedly elevated (5 U.) |
|---|---|---|---|---|
| Normal | 1.0 | 0.4-1.6 | 0 | 0 |
| Skeletal disease | | 0-1.6 | 0 | 0 |
| Hepatobiliary disease | 10.5 | 1.7-36 | 30 | 70 |
| Hepatitis with intrahepatic cholestasis | 28 | 15-35 | 0 | 100 |
| Paget's disease | 0.6 | 0.2-1.2 | 0 | 0 |

*Based on review of literature in Reference *2*.

from the same tissue. The existence of these variants has been termed "molecular heterogeneity," and the variants have been termed "isozymes." Variants may be differentiated from each other by several means—by use of chemical inhibitors, determination of Michaelis constants, thermal inactivation, electrophoretic mobility, column chromatography, immunochemical properties, and in the case of enzymes requiring co-enzymes, by kinetic studies with co-enzyme analogues (*1*). Table 6 shows the distribution of lactic dehydrogenase variants in various human tissues as determined by Vesell and Bearn (*7*). Diseases of these tissues are associated with a release of the characteristic components and are reflected in the serum by an elevation of these components.

## Biochemical Basis of Rare Disease

A number of rare conditions are characterized by deranged biochemical processes that account to a large degree for the symptomatology and course of the disease. Biochemical studies of these conditions

**Table 6.** LACTIC DEHYDROGENASE ACTIVITY IN FRACTIONS OF HUMAN TISSUE OBTAINED BY STARCH-BLOCK ELECTROPHORESIS*

| Tissue | % total activity in each peak: 1 | 2 | 3 | 4 | 5 |
|---|---|---|---|---|---|
| Heart | 0 | 0 | 3 | 24 | 73 |
| Liver | 37 | 24 | 27 | 8 | 4 |
| Skeletal muscle | 78 | 17 | 5 | 0 | 0 |
| Kidney | 0 | 2 | 11 | 45 | 42 |
| Red blood cells | 0 | 1 | 12 | 44 | 43 |
| White blood cells | 0 | 6 | 33 | 49 | 12 |

*Based on data of Vesell and Bearn (*7*).

have also resulted in the formulation of laboratory procedures that are of aid in diagnosis and management. Valid requests for such procedures are naturally infrequent. For example, the annual mortality from pheochromocytoma in the United States is about 50 (*8*). This is equivalent to a rate of about 0.028 per 100,000 population, far below most of the rates listed in Tables 1 and 2. It is unlikely that a large general hospital would have more than 1–2 such cases in a year. Yet it is imperative, both for the sake of optimal care of the patient and in the interest of the continuing education of the resident and attending staffs, that facilities should be provided for performing these determinations.

Pheochromocytomas or tumors arising from chromaffin tissue in the adrenal gland or elsewhere are characterized by excessive formation of epinephrine and norepinephrine. The biosynthesis of these two compounds is mediated through the following steps: oxidation of tyrosine to 3, 4-dihydroxyphenylalanine, catalyzed by tyrosinase; decarboxylation of 3, 4-dihydroxyphenylalanine to hydroxytyramine in the presence of a decarboxylase; hydroxylation of the $\beta$-carbon in the side chain to norepinephrine through the action of dopamine-$\beta$-oxidase; methylation of norepinephrine to form epinephrine in the presence of ATP, methionine, and an activating enzyme. The metabolism of the two hormones is accomplished by *O*-methylation at the 3-position, conjugation with glucuronic acid at the 4-position, and oxidation of the amino or methylamino group. Labeling procedures have facilitated the determination of the distribution of the various metabolites in the urine of man (*9*).

The diagnosis of pheochromocytoma has been greatly aided by the determination of epinephrine and norepinephrine in the blood and urine of man and of the urinary excretion of a major metabolite, 3-methoxy-4-hydroxymandelic acid. For example, Roth and his associates (*10*) obtained a range of 0.75–5.5 $\mu$g. and an average of 2.5 $\mu$g./L. of epinephrinelike substances of plasma in patients without pheochromocytoma and a range of 6.9–360 $\mu$g./L. in 36 patients with this condition. However, spuriously high values may be obtained in other conditions. The normal 24-hr. urinary excretion of catecholamines has been estimated to be as little as 20–40 $\mu$g. by one group and as much as 50–200 $\mu$g. by others. In patients with pheochromocytoma, values from 300 to 15,000 $\mu$g. have been reported. Thus, Gitlow *et al.* (*11*) found that the excretion of 3-methoxy-4 hydroxymandelic (vanilmandelic) acid did not exceed 3.0 $\mu$g./mg. of creatinine in normal persons

or patients with hypertension, whereas the lowest value in the group with pheochromocytoma was 6.0 μg./mg. of creatinine.

Another rare condition which has assumed considerable interest during the past decade is malignant carcinoid. Although the existence of carcinoid tumors in the gastrointestinal tract had been recognized for over 100 years, Biörck and his associates (*12*) observed in 1952 that this condition was associated with flushes, cyanosis, chronic diarrhea, respiratory distress, and valvular disease of the heart. The substance responsible for this symptomatology and secreted by the malignant carcinoid was found to be 5-hydroxytryptamine (enteramine or serotonin). The metabolic steps involved in its formation are: the oxidation of tryptophan to 5-hydroxytryptophan through the action of a hydroxylase, decarboxylation to 5-hydroxytryptamine, and the subsequent oxidation to 5-hydroxyindole acetaldehyde by an amine oxidase and to 5-hydroxyindole acetic acid (5-HIAA) by aldehyde dehydrogenase.

The urinary excretion of the end metabolite 5-HIAA has proved a most useful laboratory aid in the diagnosis of malignant carcinoid. The normal 24-hr. excretion has been determined by several groups of investigators, the values usually ranging between 2 and 9 mg. (*13, 14*). The urine contains only traces of 5-hydroxytryptamine, but the whole blood contains 0.1–0.3 μg. of this compound per milliliter, almost all of which is bound by the platelets. In patients with the carcinoid syndrome, the 24-hr. urinary excretion of 5-HIAA is well above the upper limit of normal, ranging from 70 to almost 600 mg. A few clinically atypical cases have been reported in which 5-hydroxytryptophan and 5-hydroxytryptamine, usually absent or present in only very small amounts in the urine of carcinoid patients, constitutes a substantial fraction (20–40%) of the excreted 5-hydroxyindoles (*15*).

### Hereditary Diseases

The past decade has seen the redefinition of many hereditary diseases in terms of enzymic and nonenzymic protein deficiencies. Some of the better known of these enzyme defects are: glucose-6-phosphatase in Type I glycogen storage disease of the liver and kidney (von Gierke's disease); the brancher enzyme, amylo (1, 4 to 1, 6) transglucosedase, in Type III glycogen storage disease; the debrancher enzyme, or amylo-1, 6-glucosidase, in Type IV glycogen storage disease; alkaline phosphatase in hypophosphatasia; and glucose-6-phosphate

dehydrogenase deficiency in susceptibility to hemolysis after administration of primaquine, naphthalene, or fava beans.

Some of these tissue enzyme defects are reflected peripherally in the serum, erythrocytes, or leukocytes. Analyses for these enzymes are now within the province of the clinical biochemist and can offer aid not only in the initial diagnosis of the hereditary disease, but also in following the management of the patient. Many nonenzymic protein deficiencies are also reflected in the peripheral circulation, and procedures for determining the presence of these in the blood have already been, or are in the presence of being, developed. Among these deficiencies are sickle-cell anemia, thalassemia, and other conditions characterized by abnormal hemoglobins, congenital aggamaglobulinemia, ceruloplasmin deficiency, congenital afibrinogenemia, and other dysproteinemias and clotting-factor deficiencies. Electrophoresis, immunochemistry, and technics combining the features of both may be applied as aids in the diagnosis of these diseases. For hereditary diseases not reflected in the peripheral circulation, ultramicrochemical methods for tissue analysis, now being developed in such laboratories as those of Glick (*16*) and Lowry (*17*), may also have to be utilized by the clinical biochemist.

## The Discord between the Clinician and the Laboratory

In spite of the contributions of biochemistry to medicine, contributions which are generously acknowledged and even praised by the clinician, he retains, as noted above, a general air of reservation about the specific results which the clinical biochemistry laboratory submits. Most of us who, either as biochemists or clinical pathologists, have had the responsibility of directing such a laboratory, have been concerned by this sense of reservation.

The reasons for discord between the clinician and the clinical biochemistry laboratory on this point are, I believe, due to several factors: the type of training the physician receives, the nature of diagnostic biochemical determinations, and, finally and regrettably, the history of lack of precision in many clinical laboratories.

The clinician has been trained from his student days to place great reliance on the information that his senses bring him. What he sees of the patient either directly or through his ophthalmoscope, otoscope, or similar instrument; what he hears on percussion or through his stethoscope; what he feels on palpation; and what, in a bygone age, he smelled or tasted, seem to him most cogent factors in evaluating the

state of his patient, either for purposes of diagnosis or in following treatment. His own experiences along these lines are integrated with his colleagues' experiences as these are transmitted personally or through the literature and, in a general way, with the vast body of correlated laboratory data accumulated during the past 50 years.

But in the specific instance, what is done in the diagnostic laboratory or department is geographically remote and, in its details, is largely unfamiliar to the clinician. The skeptical clinician may sit down with the pathologist to review a slide from a biopsy or with the hematologist to examine a blood smear. He may modify his clinical impression by having the roentgenologist point out on an X-ray plate a lesion of which he was unaware. But the microbiologist and the biochemist deal with evanscent biologic materials which it would be impractical to store and reanalyze again in the presence of the clinician. The biochemist has only a secondary record of his work which must be accepted or discarded by the clinician at face value.

The history of poor technical performance in many clinical laboratories has also tended to depreciate the role of the diagnostic biochemistry laboratory. During the past 15 years, several clinical biochemists and clinical pathologists have noted with increasing uneasiness the great variability obtained by different clinical laboratories in the analysis of known prepared solutions, sera, or stabilized bloods (*18–21*).

The existence of these surveys indicates the growing awareness of the problem of interlaboratory variability and possible error. Thirty to forty years ago, a greater proportion of professionally trained biochemists were interested in the clinical aspects of their field than is now the case. Rare, however, was the hospital that employed a biochemist to supervise the diagnostic biochemical laboratory. In many hospitals and even medical and university centers, supervision and control were inadequate. The precision and validity of laboratory determinations were like the fate of a rowboat upon the open sea.

It is not surprising, therefore, that except in a few special hospitals, the clinician had little trust in laboratory determinations. If the results agreed with his clinical impressions, he accepted them. Otherwise, he discarded them. Indeed, there arose the clinical maxim, "If a laboratory report does not agree with the clinical impression, then no attention should be paid to the report." I believe that this attitude is becoming increasingly unwarranted today. It is not in the best interests of good medical care or of the proper teaching of the medical student, intern, or resident to perpetuate this attitude.

## Reconciliation of the Clinician and the Clinical Laboratory: The Role of the Laboratory

Before the clinician can be persuaded that he should not wave aside the results of the clinical laboratory when these are in disagreement with his provisional clinical evaluations, he must become utterly convinced of the precision of the determinations, whether these come from his hospital laboratory or from some other source.

As a result of their survey of clinical laboratories in Pennsylvania in 1946, Belk and Sunderman (*18*) reported a wide variability in the determination of prepared aqueous solutions of various components. For example, a solution of 6.6 mg. of calcium per 100 ml. was analyzed by 43 laboratories and yielded results ranging from 3 to 17.2 mg./100 ml. Only 14 of the 43 results fell within an experimental error of ± 0.5 mg. or ± 8%, and 26 of the results fell within an error of ± 1.0 mg. or ± 16%. Similarly, a prepared glucose solution of 60 mg./100 ml. yielded results ranging from 40 to 571 mg./100 ml., and only 27 of 52 laboratories gave results between 55 and 65 mg./100 ml.

Interlaboratory variability may be due in part to the use of different methods with varying degrees of specificity for the component being analyzed. However, Wooton and King (*19*) found that even when the same method was used by the different hospital laboratories on a single specimen of preserved blood, considerable variability was still obtained: 40–180% of the theoretical concentration for urea, 35–170% for nonprotein nitrogen, 35–175% for uric acid, 65–145% for cholesterol, and 60–135% for chloride. The variability was less in the case of known prepared solutions. It is not clear from these reports whether the analyses were conducted under "blind conditions" or whether the technicians were aware that they were being tested.

The reasons underlying the existence of interlaboratory variations and the presence of the errors which these imply require consideration. The true value $t$ of any chemical characteristic may be expressed generally by the equation $t = m \pm a \pm C$, where $m$ is the mean of the observed values, $a$ is the random or experimental error, and $C$ is the constant or systematic error.

The class designated as random errors refers to those encountered by a single observer in making successive measurements under stated circumstances, e.g., performing replicate determinations of the concentrations of glucose, urea, or other biochemical component in the same blood sample. These random errors may be due to subjective fac-

tors such as fluctuation in the observer's attention or state of fatigue or to such objective factors as variations in volumes of pipets or changes in ambient temperature.

The extent of the random error and hence the precision of the mean value for successive measurements or replicate determinations may be expressed in any of several ways. Thus the average error is defined as the average of the deviations $d$ (without sign) of the deviations from the mean value $m$ that is, $a = \frac{\pm \Sigma(d)}{n}$ where n is the number of observations. The percentage average error is $a\ (100)/m$. The standard deviation is expressed as S.D. $= \pm \sqrt{\frac{\Sigma(d)^2}{n-1}}$ and the probable error as $p = \pm 0.675 \sqrt{\frac{\Sigma(d)^2}{n-1}}$.

The extent of random errors in diagnostic biochemistry determinations may be briefly illustrated. In setting up their method for the determination of cholesterol, a complex method that involves a considerable number of steps, Schoenheimer and Sperry (*22*) analyzed each of 3 sera in triplicate and 2 sera in quadruplicate. The average error ranged from ± 0.44 to ± 1.2% of the mean, and the average of these errors was 0.9%. It is the practice in our laboratory to determine daily in duplicate the cholesterol concentration of a sample of a large serum pool by a slight modification of the method of Schoenheimer and Sperry. We find that these duplicate determinations usually check within anywhere from ± 0.0 to ± 1 5%, averaging about ± 0.6%—precision of the same order as that obt ined originally by Schoenheimer and Sperry. Results in our laboratory as well as in others show that replicates of most clinical biochemical procedures can achieve a random error of no more than ± 2%. In other words, the error equation becomes $t = m \pm 2\% \pm C$.

The constant error $C$ cannot be detected or evaluated by mere repetition of measurements. Incorrect weighing of a compound or the unwitting use of an impure sample in the preparation of a stock standard solution may lead to an error which will persist for weeks or months until a new standard solution is prepared. Incorrect dilution of a stock standard solution, the incorrect use of pipets, and use of defective pipets or volumetric flasks are some of the other sources of constant errors.

These sources are insidious and may occasionally be fantastic. Several years ago in our laboratory, determinations of urea by the urease method began to yield somewhat higher values for the control solution, as well as the control and other bloods. Review of the procedure re-

vealed that the technicians had without warrant begun to use a high-grade filter paper instead of ordinary Whatman paper for filtration. The higher values coincided with the delivery of a new batch of filter paper in which the manufacturer had introduced the procedure of following the acid washing by a wash with an ammonia water. Another incident is also illustrative. About 2 years ago the values for the prothrombin times began to run higher by a second or two, and it was discovered that this coincided with the installation of an air-cooling system in the laboratory, with one of the outflow vents placed near the 37° water bath used for the determination.

The circumstances that lead to random and constant errors tend to be aggravated in the diagnostic clinical laboratory. In our present large hospitals and medical centers, the cost of patient care has become a pressing problem. Determinations must be set up in batches done on a "belt-line" basis and completed by the end of the day. The technician who actually performs the determinations becomes a crucial factor in assuring accuracy and precision of the results. A sense of urgency pervades the day's work. Yet the performance of a specific technic day after day and, in some laboratories, month after month, is essentially a boring business, and boredom may easily lead to error. Competent, interested technicians are difficult to obtain because of the competition presented by the vast field of research activity in this country. The latter offers a more reflective pace of work, variety in methods and procedure, opportunities to be informed about the significance of the daily work, and the general feeling of ennoblement that goes with being engaged in "research" rather than "routine" endeavors. Although it is not within the scope of this paper to consider this aspect in any detail, it is the writer's opinion that the lot of the technician in the diagnostic laboratory should be improved to match the situation in the research laboratory.

It is, of course, easy to state that random errors may be minimized and constant errors avoided by the exercise of sufficient care. But, as with other virtues, the general formulation requires specific measures to become effective. The careful and conscientious clinical biochemist or clinical pathologist attempts to gain assurance concerning the accuracy and precision of determinations in his laboratory through a varied array of technical devices. These include the institution of directions for the preparation of blood collecting tubes, the washing of glassware, the prevention of chemical contamination of flasks, pipets, and other apparatus during manipulation, and the careful preparation

and checking of standards and reagents. Weighing of compounds for preparation of standard solutions is recorded and reviewed by the meticulous biochemist. A new standard solution is analyzed against the former standard. The careful biochemist subjects "synthetic unknown," "recovery" solutions, and control sera on bloods to analysis simultaneously with each set of determinations, and when possible, has his technicians repeat all analyses that have yielded values outside of the normal range.

The biochemist will perform other analyses in replicate to determine the extent of random error in his laboratory. It is not an uncommon experience to find that duplicate determinations performed on the same day yield a mean value which differs by more than the usual random error from the mean of a pair of duplicate determinations on a subsequent day. Thus the mean values of duplicate determinations of the cholesterol concentrations in specimens of one serum pool on 20 successive laboratory days yielded deviations ranging from $-$ 6.8 to $+$ 7.5% from the over-all mean value and an average error of $\pm$ 3.7%, greater than the average error, $\pm$ 0.6%, of replicate determinations on the same day. The greater error of $\pm$ 3.7% represents an exaggeration of the factors that enter into the causes of random errors.

The emergence of constant errors may be detected by scrutinizing each day the distribution of the results obtained for the analysis of the various components. Most of the determinations in the clinical biochemistry laboratory of a hospital are likely to be within the normal range. Any shift of the range toward high or low values should indicate the emergence of a constant error, and due measures for its correction can then be taken.

## Cooperation between the Clinician and the Laboratory: The Role of the Clinician

This cooperation involves not only a recognition of the currently intense efforts that the clinical biochemist and pathologist are making at self-criticism and improvement of laboratory performance, but also a reorientation of attitudes toward the relationship between laboratory and clinical medicine. It should be recognized that the precision and accuracy of a laboratory determination are the resultant of many steps which begin outside the laboratory, namely, at the vein of the patient. The physician should not choose to disregard any of these steps, however trivial they may seem to him, with the expectation that he will be

able to judge their importance and the verity of the final analysis in the light of the patient's clinical condition. Such disregard may lead to error that is not within the power of the laboratory to control and to bias of clinical judgment.

Nor should the clinician disregard the drawing of blood under fasting or other basal conditions, where this is indicated, for such disregard tends to blunt the distinction between the normal and abnormal ranges of values. When the drawing of blood is within the physician's supervision, he should inform the nurse, student, intern, or resident of the necessity of using specially prepared, chemically clean and dry, as well as sterile syringes and needles, chemically clean and capped containers for blood or other materials, and a standardized volume or amount of anticoagulant and antiglycolytic materials. The availability of specially prepared vacuum tubes and disposable needles renders this caution less necessary today. It is obvious, of course, that the clinician be aware that intravenous or other therapy may influence greatly the results of laboratory analyses. Important too is the constant watchfulness for contamination of the needle by solutions being readied for intravenous injection while a blood is being drawn for a diagnostic purpose. Because most laboratory procedures are essentially microchemical, contamination with a single drop of a therapeutic solution may produce very large errors.

However vigilant the biochemist may be concerning the sources of error, there will still be instances of seeming discrepancy between the laboratory result and that expected on the basis of the patient's condition. Such discrepancies should not be relegated to a mystical realm or quickly labeled as a laboratory error but should be immediately evaluated in terms of both laboratory procedures and clinical considerations. Cooperation in such evaluation will, I believe, be of advantage both to the clinician and the clinical biochemist. I should now like to give several specific examples of such cooperation.

The laboratory was informed immediately that a low serum potassium, about 2 mEq./L., had been reported when the clinical condition did not seem to indicate hypokalemia. Upon review of the laboratory procedures, the biochemist ascertained that the laboratory aide had placed several 3.0-ml. pipets in the drawer reserved for 2.0-ml. pipets. The possibility arose that 3.0 ml. instead of 2.0 ml. of 0.3N lithium sulfate had been used in diluting the particular serum for a flame-photometric determination. That this indeed had occurred was demonstrated by determining the dry weight of an aliquot of the first dilution

of the serum and by repeating the analysis on the very same serum, diluted now with 2.0 ml. of the lithium sulfate solution.

The second example illustrates the manner in which chemical contamination can create an apparent discordance between the laboratory and the clinician. A child clearly had all the clinical signs of tetany, but the serum calcium determination, instead of yielding a value of 5–6 mg./100 ml., was higher than normal. Review of the conditions under which blood had been taken by the resident indicated that he had been about to inject a 10% calcium gluconate solution but had decided to take blood first. A small and presumably negligible drop of calcium gluconate might have been left on the skin before venipuncture. Such a drop contains 0.5 mg. of calcium, equal to the amount present in 5 ml. of normal serum and great enough to have accounted for the doubling of the analytical value. Analysis of a blood taken under proper conditions indicated a calcium level of about 6 mg./100 ml.

It has been said that in science one learns more by paying attention to findings that do not fit in with his theory or preconception. If the clinician can depend completely on the diagnostic biochemical laboratory, he is in a position to consider thoughtfully apparent discrepancies between his clinical formulations and the laboratory, to re-examine these formulations, and to add further to his own clinical knowledge. This situation is illustrated by the following example.

A 52-year-old male was found at operation to have a bronchogenic carcinoma with metastases. During the next 2 years evidence of intracranial metastases developed. A lumbar puncture revealed increased pressure, a protein concentration of 263 mg./ml., a cell count of 170, and a glucose concentration of 2 mg./100 ml. The latter determination, because it was so abnormally low, was repeated and duplicated in the laboratory on the same specimen of spinal fluid. The value was considered as possibly erroneous, since cultures and smears of the fluid were negative for pyrogenic or other organisms. The biochemist pointed out that although almost all instances of low spinal fluid glucose were due to the action of organisms, the possibility existed that the neoplastic involvement of the meninges might also lead to low concentrations of glucose in the spinal fluid, either through an effect on the permeability of the blood—brain barrier or because of excessive metabolism of glucose by the neoplastic tissue. Indeed, reports by Madow and Alpers (*23*) in 1 case, by Dodge *et al.* (*24*) in 4 cases, and by McCormack and associates (*25*) in 6 cases had indicated that the spinal fluid sugar might range from 0 to 25 mg./100 ml. in patients with

diffuse neoplastic involvement of the meninges. This information indicated that the patient under discussion fell into this group. Autopsy and microscopic findings confirmed this indication.

A fallacy shared alike by some clinicians and by chemists not acquainted with diagnostic biochemistry is that the precision of a determination need be only as good as the biologic variability of the components under analysis. Why try to determine whether the concentration of blood glucose is 80 or 83 mg./100 ml. when the normal range (mean ± 2 standard deviations) is anywhere from 70 to 110 mg./100 ml.?

The way in which the concentration of various components in the blood varies in any one individual has been of intermittent interest for many years. For example in 1920, Hammet (*26*), impressed by Cannon's observations of physiologic reactions in animals to various emotional stimuli, studied the extent to which the variability of the concentration of such blood components as total nitrogen, nonprotein nitrogen, creatinine, uric acid, and the like, was related, in the same individual over a period of several weeks, to his emotional status (*26*). In the subsequent 10–15 years, a number of other psychologic and psychiatric studies were carried out along these lines (*27*). More recently, Roger J. Williams (*28*) submitted the thesis that even though normal individuals show variability in biochemical and physiologic characteristics, the points about which variations occur for any stated characteristic may differ distinctly from one individual to another within the normal group. George Z. Williams (*29*) documented this with regard to several blood components and pointed out the implications for studies in preventive medicine and incipient pathologic alterations. We have also had the opportunity to observe in our laboratory the distinction between intra- and interindividual variability in normal persons. It is our custom to perform various blood biochemical analyses at half-year to yearly intervals on our presumably normal group of technicians. We have found that in some, values oscillate within a range of 10–20 mg. about a serum cholesterol concentration of 130 mg./100 ml., whereas in others the values fluctuate about a level of 220 mg./100 ml.

The following example (*3*) illustrates how changes in a biochemical component, in this case, serum calcium, though still "normal," can herald changes in the clinical condition. The patient was a 42-year-old woman who had been operated on 3 years previously for carcinoma of the breast and who subsequently developed metastases. During a period of observation in the hospital when she was in a relatively good condition, she had several serum calcium determinations performed at

intervals of 2–3 days. These ranged between about 9.2 and 10.0 mg./100 ml. The mean normal value by the method in our laboratory is 10.0 mg./100 ml., with a standard deviation of ± 0.4 mg., or a normal range of 9.2–10.8/100 ml. Within a few days, the serum calcium began to climb from a value of 9.5 mg. to 9.8 mg./100 ml., and then to 10.1 mg./100 ml. These values were all within the normal range, but this climb was the precursor of values that 4 days later clearly passed into the lower hypercalcemic range, to 11.4 mg./100 ml. The clinical condition now worsened, was marked by nausea, anorexia, vomiting, and bone pain, and was associated with serum calcium values climbing steadily from 11.4 to 13.7 mg./100 ml. and to abnormally high urinary excretions ranging from about 500 to 700 mg. per day.

## Summary

In this paper we have attempted to define the relationships between clinical medicine and diagnostic or, as it is more frequently termed, clinical biochemistry. We have indicated first, the role that general biochemistry has played in elucidating mechanisms of disease and in providing the clinician with procedures that aid him in the diagnosis of disease and in the care of the patient. We have illustrated this role by reviewing very briefly some of the recent contributions such as the expanded diagnostic use of serum enzymes; the correlation of subcellular structure in human tissues with function at enzymatic levels; the investigation of enzyme variants; the metabolism of epinephrine and norepinephrine in pheochromocytoma; the metabolism of tryptophan in malignant carcinoid; and enzyme defects in hereditary disease.

We noted the reservations with which the clinician frequently views the specific results that he obtains from the diagnostic biochemistry laboratory and have attempted to analyze the bases for these reservations. In this connection we considered the phenomenon of interlaboratory variability, the nature of the random and the constant errors that may exist within a laboratory, and the measures that the clinical biochemist may take to counteract these errors.

We have tried to indicate the steps the clinician may take in a fuller and more knowledgeable utilization of the data from the diagnostic biochemistry laboratory. These involve a recognition of the efforts and advances that are being made in increasing precision and accuracy within the laboratory and the role that the clinician himself can play in contributing to this precision and accuracy. The phenomenon of intra-

individual and interindividual variability of biochemical parameters, and the significance of this phenomenon in diagnosis, are also worthy of the clinician's attention. Finally, the clinician should abjure the concept that a seeming discrepancy between his formulation and laboratory results usually means a laboratory error. Thoughtful review, in cooperation with the biochemist, of such discrepancies are of value both for clinical medicine and clinical biochemistry.

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# The Renal Aminoacidurias

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THE FINDING of increased amounts of amino acids in the urine is characteristic of several inherited as well as acquired metabolic diseases. Such aminoacidurias are usually classified as "overflow" or "renal" in origin. In the former instance, it is assumed that the concentrations of amino acids in the plasma markedly exceed the capacity of the normal renal tubule to reabsorb completely the filtered load of amino acids. In all such cases, the quantities of amino acids in the plasma will be found to be in excess of the normal. Examples of such "overflow" aminoacidurias are those found in far-advanced liver disease and also in phenylketonuria. However, the majority of aminoacidurias of clinical interest are of the renal type, in which, even with normal concentrations of amino acids in the plasma and, consequently, normal filtered loads in the kidneys, there are increased amounts of amino acids excreted into the urine. Under these circumstances the cause of the aminoaciduria is attributed to a decreased capacity of the renal tubule to reabsorb amino acids. To appreciate fully the significance of a renal aminoaciduria, it is necessary to review the normal mechanisms whereby the renal tubule handles amino acids. Failure to do so has led to erroneous interpretation of the results of analysis for amino acids in the urine.

## Normal Renal Reabsorption of Amino Acids

In normal subjects under fasting conditions, there is almost complete reabsorption of all of the amino acids filtered at the renal glo-

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merulus. If a mixture of amino acids is infused intravenously so that the filtered load is increased, then both the amounts of amino acid excreted and those reabsorbed are increased. As a result, it would be expected that the attainment of a maximum tubular reabsorptive capacity (Tm) would be gradual. A Tm has been demonstrated for certain amino acids in the dog (*1*), but attempts to do so in the human were unsuccessful. In the experiments of Doolan *et al.* (*2*), it was believed that it might be possible to reach a Tm for glycine in the normal human subject. For this purpose the amino acid was infused intravenously at rapid rates to increase the filtered load. Although there were indications of an approaching Tm, the toxicity of the amino acid at such high plasma levels made it necessary to terminate the experiment before a Tm could be established.

There is evidence for competition for reabsorption between certain groups of amino acids such that a high filtered load of certain amino acids presented to the tubule for reabsorption may increase the excretion into the urine of other amino acids. An example is the effect of a high filtered load of lysine to increase the excretion of cystine (*3*).

The pattern of amino acids in the urine does not resemble that of the plasma. Thus it is clear that the renal tubule exhibits selectivity with respect to the reabsorption of each of the amino acids presented to it. Endogenous clearances of individual amino acids in humans were found by Doolan *et al.* to range from a low of 0.12 ml./min. for valine to highs of 5.12 for glycine and 6.38 for histidine (*4*). It is evident from these data that reabsorption of amino acids by the renal tubule is normally in excess of 93% of the filtered load. This is so even after intravenous infusion of free amino acids at a rate approximating that used clinically. Under these circumstances, the total amount of amino acid nitrogen excreted represents less than 4% of that given (*4*). Despite definite and in some instances marked elevations in serum concentrations, only five naturally occurring amino acids were ever excreted at a rate exceeding 0.5 mg./min., namely, glycine, histidine, serine, threonine, and occasionally lysine.

The reabsorption of amino acids by the renal tubule is thought to be a process of active transport involving a mechanism of a highly selective type. This idea is supported by the fact that the efficiency of reabsorption of the naturally occurring L isomer of an amino acid is much greater than that of the D isomer of the same amino acid (*5*). The observation that a significant increase in clearance of the D form follows a diuresis induced by a water load is taken as consistent with the

notion that the D forms are either passive participants in or committed to a different transport mechanism than that associated with the natural L form. Differences in rates of transport of D and L forms of amino acids have also been shown to be characteristic of the intestinal epithelium and of the human placenta (*6*).

From what has been stated above, it should be apparent that the excretion of amino acids is normally influenced by the glomerular filtration rate, even when the plasma concentrations of amino acids are normal. If one is interested in establishing that an aminoaciduria is of the renal type, that is, attributable to a renal tubular defect, it thus becomes necessary to take the glomerular filtration rate into account. An example of the importance of this fact is to be found in the circumstances which surround the apparent aminoaciduria of normal pregnancy. In this connection, a histidinuria has attracted the most attention, presumably because histidine is normally cleared in comparatively large amounts and therefore can be readily detected even by a colorimetric method. In fact, the so-called histidinuria of pregnancy is so characteristic that it was proposed as a chemical test for pregnancy (*7*) at a time when the modern tests had not been devised. It was further observed that the expected histidinuria disappeared in pregnant subjects with preeclampsia. It would be expected that various metabolic causes not involving simple renal mechanisms would be invoked to explain these phenomena. Particularly attractive because of the absence of the normal histidinuria was the idea that an abnormality in histidine metabolism, perhaps involving excess formation of histamine, was involved in the etiology of the preeclamptic state.

Studies of renal clearance of histidine in normal pregnancy and in pregnant subjects with preeclampsia by Page *et al.* (*8, 9*) revealed that in normal pregnant women, the rate of glomerular filtration increased from a nonpregnant mean of 104 to a pregnancy mean of 171 ml. per minute per 1.73 sq. M. of body surface. It was this change in glomerular filtration rate, aside from any change in tubular reabsorption or decrease in rate of metabolism of histidine, that accounted for about half of the excess histidine excreted. In contrast, in the preeclamptic patients, there was a marked diminution in glomerular filtration rate that was largely responsible for the decreased excretion of histidine.

It should be obvious that the existence of an aminoaciduria may be overlooked in patients with a marked decline in glomerular filtration rate as indicated by uremia and as conveniently measured by creati-

nine clearance. An important illustration of this situation is to be found in following the course of a young patient with the generalized aminoaciduria and other renal tubular defects of the Fanconi syndrome. Early in the course of the disease, when the glomerular filtration rate is still relatively normal, the generalized aminoaciduria, which is due to a renal tubular reabsorptive defect, is readily apparent. However, as the disease progresses over a period of several years, it will be noted that the excess excretion of amino acids in the urine declines to normal. This is concurrent with a reduction in the glomerular filtration rate, as evidenced by a rising blood urea nitrogen and other signs of uremia. Indeed, in these patients, shortly before death occurs as a result of renal failure, there may no longer be any evidence of significant aminoaciduria. It follows that if such a patient were to be studied for the first time at this late stage of the disease, a negative diagnosis of generalized aminoaciduria would be forthcoming.

## Diseases Associated with Aminoaciduria

With the above facts concerning the renal handling of amino acids in mind, we may now turn to a consideration of some of the specific diseases in which a renal aminoaciduria is an associated biochemical finding. In some instances the aminoaciduria is generalized, that is, increased excretion of all of the amino acids occurring in the plasma can be detected. In other instances, the aminoaciduria is more specific in that there are increased amounts of some amino acids in the urine while all others are excreted in normal amounts. It should also be noted that the aminoacidurias may result from an inherited metabolic abnormality, in which case the aminoaciduria is a permanent finding, or it may be an acquired abnormality which may either be transient or become permanent. Examples of transient aminoacidurias are those reported as occurring during the diuretic phase after acute renal insufficiency, or as a result of a deficiency of potassium. Aminoacidurias of longer duration occur as a result of poisoning with heavy metals, particularly cadmium and uranium, but also with lead or mercury. The aminoaciduria often found in patients with Wilson's disease may also be included here because it appears to be associated with the toxic effect on the renal tubule of copper, which characteristically accumulates in patients with this disease. Generalized aminoacidurias are also occasionally found in association with the nephrotic syndrome (*10, 11*) and with multiple myeloma (*12, 13*).

### Fanconi Syndrome

The Fanconi syndrome is perhaps the most frequently studied among the genetically transmitted aminoacidurias (*14*). The syndrome as recognized in infants and children is characterized not only by a generalized aminoaciduria but also by other renal tubular defects affecting reabsorption of phosphate and glucose, and frequently also the renal handling of potassium and water, as well as the secretion of hydrogen ions and the manufacture of ammonia. The aminoaciduria itself, although generalized, is quantitatively minimal and is therefore of no metabolic consequence. This is in contrast to the significance of the other tubular abnormalities. Thus, the persistent phosphate leak engenders a negative phosphorus balance and resultant impairment of development of bone, as evidenced by the occurrence of rickets. The serum phosphate is generally lower than normal, and vitamin D in ordinary dosages is therapeutically ineffective. This form of renal rickets is in consequence often described as hypophosphatemic or vitamin D–resistant rickets. The other associated renal tubular abnormalities affecting the handling of electrolyte and water and the renal contribution to acid–base balance are also of serious import. It is therefore apparent that although a generalized aminoaciduria may be of no particular metabolic significance in itself, it is of value to suggest the necessity of further studies to assess the full extent of the renal tubular defect. There is also the important fact that these generalized renal aminoacidurias are often found in association with other inherited abnormalities such as cystine storage disease (cystinosis) (*15*), galactosemia (*16*), or diseases affecting mental development, as in Lowe's syndrome (*17*).

Manifestations of the Fanconi syndrome may also be observed in adults (*18*). Although usually these are acquired as a result of concurrent involvement of the kidney by a pathologic process, there is evidence that in some instances the adult manifestation of the syndrome is on an inherited basis. The consequences of the renal tubular phosphate leak are most prominent in these cases. Here the defect is recognized as a form of osteomalacia (the analogue of the renal rickets described in the childhood form of Fanconi disease). It is believed that this was actually the cause of the osteomalacia in the case described by Milkman (*19*) and of the similar cases of "idiopathic" osteomalacia subsequently described and eponymically designated "Milkman's syndrome."

### Cystinuria

The aminoacidurias characterized by the excretion of relatively few amino acids are best illustrated by the inherited disease of renal tubular transport usually referred to as cystinuria. In this disease four amino acids—cystine, lysine, arginine, and ornithine—are excreted in large quantities. Indeed, from clearance studies (*20*, *21*), which indicate that the clearance of several of the affected amino acids approaches that of inulin, it has been concluded that the renal tubular mechanism involving transport of these four amino acids is virtually inactive. No deleterious metabolic result follows the conspicuous aminoaciduria of cystinuria except the likelihood of the occurrence of cystine calculi and consequent renal damage attendant upon the inability to keep in solution such large quantities of cystine as are present in the urine of the cystinuric patient.

### Glycinuria; Hartnup (H) Disease

More specific manifestations of defects in renal tubular transport for amino acids are exemplified by glycinuria (*22*) and the aminoaciduria of Hartnup (H) disease (*23*), although in these diseases the metabolic defect may well be much more generalized. For example, in patients diagnosed as exhibiting H disease, dermal symptoms may be present, consisting of an intermittent, red, scaly, pellagra-like rash appearing after exposure to sunlight, and there may also be attacks of cerebellar ataxia as well as psychiatric changes. However, the renal aminoaciduria is a constant feature, and indeed this is the most reliable diagnostic feature of the disease. The aminoaciduria is not generalized, but its pattern is very characteristic. There is a significantly increased excretion of alanine, asparagine, glutamine, histidine, isoleucine, leucine, phenylalanine, serine, threonine, tryptophan, tyrosine, and valine. The excretion of the other naturally occurring amino acids remains normal. It is of interest that in the aminoaciduria of H disease, none of the four amino acids excreted in greatly increased amounts in cystinuria is involved.

Glycinuria has been recognized in 4 female members of three generations of a single family. Although experience with this aminoaciduria is still limited, it would appear to be attributable to a selective renal tubular defect in glycine transport. However, 3 of the glycinuric subjects also had a nephrolithiasis due chiefly to calcium oxalate. The fact that glycine is a metabolic source of endogenous oxalate may indicate

that glycinuria has other metabolic manifestations in addition to that affecting the renal tubular reabsorption of glycine.

It must be evident that a continuing study of the aminoacidurias, made more expeditious by the recent introduction of improved technical methods, will prove increasingly fruitful in the elucidation of disease entities of obscure etiology.

## Summary

The normal mechanisms whereby the renal tubules handle amino acids are reviewed as a basis for interpretation of the physiologic causes of the renal aminoacidurias. Under normal circumstances, the renal tubules reabsorb in excess of 93% of the amino acids filtered from the plasma. When the filtered load of amino acids is increased, as by intravenous injection of amino acids, there is an increase in both the amounts reabsorbed and those excreted, but the ability of the renal tubule to respond to an increased filtered load of amino acids is so great that a maximum rate of reabsorption has not been found in the human. However, the tubule does exhibit selectivity with respect to the reabsorption of each of the naturally occurring L-amino acids; furthermore, there is a much greater efficiency of reabsorption of the L as compared to the D isomers of the individual amino acids.

The excretion of amino acids is influenced to an important degree by the glomerular filtration rate, as exemplified by studies in pregnant subjects as well as patients with renal disease affecting glomerular filtration.

Several renal aminoacidurias of clinical interest are discussed with special reference to their diagnostic and metabolic significance.

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# Determination of Serum Amino Acids

*Robert D. Rapp*

**The reaction of 1-fluoro-2,4 dinitrobenzene with amino acids was investigated as an assay procedure for serum amino acids. An aliquot of a tungistic acid filtrate of serum is buffered at *p*H 10, mixed with the buffered reagent, and heated at 56° for 15 min. The resultant color is read at 420 mμ.**

ALTHOUGH several procedures are available for the determination of amino acids in biologic material, they are time consuming and laborious. The reaction of 1-fluoro-2,4-dinitrobenzene with amino acids was investigated as potentially a more direct procedure. This reaction has been employed for terminal amino acid analysis in protein degradation studies by Sanger (*1*). It also has been used as the basis of an analytic procedure for amines by Dubin (*2*). The method of Dubin however is unsatisfactory for the determination of amino acids in serum because of the presence of protein.

## Experimental

### Reagents

*1-Fluoro-2,4-dinitrobenzene* (Eastman-Kodak No. 6587) This compound may be stored for months in a refrigerator. To prepare the stock reagent, 0.65 ml. is dissolved in 50 ml. of acetone. The stock reagent is satisfactory for at least 3 months when refrigerated. For use, 0.1 ml. of stock reagent is mixed with 1.0 ml. of borate buffer, 0.1 M, *p*H 10.0. This solution is very unstable and must be prepared immediately before using.

*Borate buffer 0.1 M, pH 10.0* To prepare, 38.1 gm. $Na_2B_4O_7 \cdot 10 H_2O$ is dissolved in 750 ml. of distilled water. Concentrated alkali solution (50% w/v NaOH) is then added to bring the *p*H to 10.0, and the solution is diluted to 1000 ml. The *p*H is determined with a Beckman model G *p*H meter.

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*Sodium tungstate 10%* To prepare, 10.0 gm. sodium tungstate is dissolved in 80 ml. of distilled water. The solution is diluted to 100 ml. with distilled water.

*Sulfuric acid N/12*

*Hydrochloric acid, concentrated*

### Procedure

A Folin-Wu protein-free filtrate is prepared using 1.0 ml. of serum, 8.0 ml. of N/12 sulfuric acid, and 1.0 ml. of 10% sodium tungstate. One milliliter of the filtrate is mixed with 1.0 ml. of 0.1 M borate buffer and 0.1 ml. of dilute buffered 1-fluoro-2,4-dinitrobenzene solution added. The reaction mixture is heated in a 56° bath for 15 min., after which 1.0 ml. of distilled water and 0.1 ml. of concentrated hydrochloric acid is added. The contents are well mixed and read in a spectrophotometer at 420 mμ using distilled water as a blank.

## Results and Discussion

In order to arrive at optimum conditions for the determination, the effects of temperature, time, and *p*H were studied. The reaction was rather interesting as it exhibited zero-order kinetics. This is indicative of a catalytic reaction. The slopes of the resultant curves showed good reproducibility while the intercepts did not. The curve showing change in absorbance (O.D.) with respect to time had a mean slope of 0.0103 change in O.D. per minute (Fig. 1). This corresponds to a change of 0.3 mg. N/100 ml./min.

The study of the *p*H requirements showed the reaction to have a first-order dependence on hydroxyl ions. This indicated that the rate-determining step involves the hydroxyl ion. The mean slope of this curve was 0.0628 change in O.D. per *p*H unit, which is equivalent to approximately 1.8 mg. N/100 ml. of sample (Fig. 2). This clearly demonstrated the need for adequate buffering of the reaction media. A mechanism consistent with the above observation would involve the attachment of the amino nitrogen to the aromatic carbon bearing the fluorine atom. Because of the difference in electronegativity between the carbon and fluorine atoms, the bond would be polarized to an appreciable extent. The ionic character of the bond would be further increased by the electron-withdrawing properties of the two nitro groups on the ring. This is supported by the ease with which the compound is hydrolyzed to dinitrophenol. After attachment of the amino nitrogen to the polarized carbon atom, the fluorine atom is

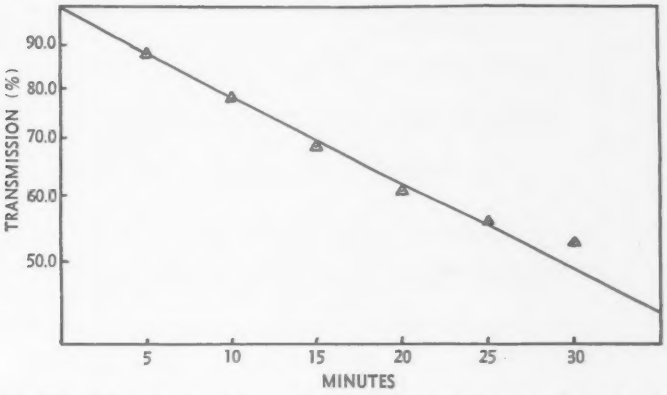


**Fig. 1.** The relation of per cent transmission to time for glycine at a *p*H of 9.0 and 56°.

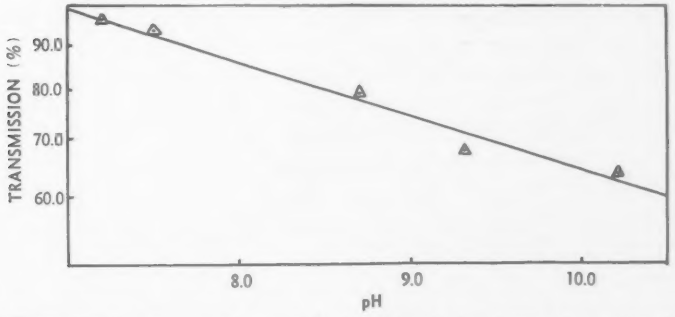


**Fig. 2.** The relation of per cent transmission to *p*H for glycine at 56° and a reaction time of 10 min.

probably lost as a fluoride ion, yielding a positively charged species. The abstraction of a hydrogen ion by the hydroxyl ion would then produce the dinitrophenyl amino acid. It is this last step, according to the kinetic data, that would determine the rate.

The reaction also shows a linear relation with temperature. Here the O.D. changed by only 0.0028 per degree centigrade (Fig. 3). The curve follows the Arrhenius equation closely, but activation energies were not computed.

In view of the results described above, a temperature of 56°, a *p*H of 10.0, and a 15-min. reaction time were used. The temperature of 56° was chosen because most clinical laboratories would be equipped

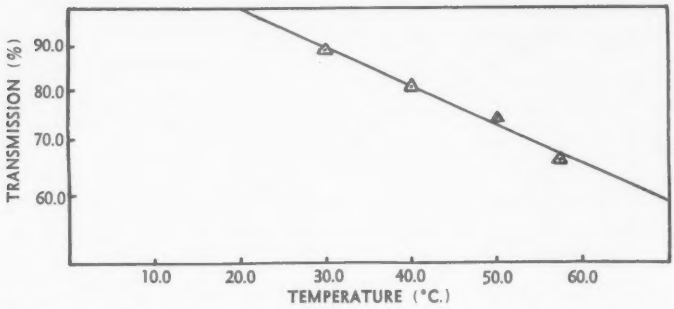


**Fig. 3.** The relation of per cent transmission to temperature for glycine at a *p*H of 10.2 and a reaction time of 10 min.

with a bath providing this temperature. Since small variations of time and *p*H in particular can cause variations in results, the use of a standard is recommended. It is also best not to make more than six determinations at one time.

The reaction was checked to determine if creatinine, urea or uric acid might interfere. These substances exhibited no reaction under the conditions employed. This is in contrast to the method of Frame *et al.* (*3*), where a correction for uric acid interference is necessary. The standard deviation and probable error for this determination were obtained using a glycine solution equivalent to 1.2 mg. N/100 ml. of serum. The standard deviation was 0.052 mg./100 ml., and the probable error, 0.035 mg./100 ml. Normal values obtained from 20 selected patients ranged from 3.4 to 6.0 mg./100 ml. of serum with 60% of the values occurring between 4 and 5 mg./100 ml.

This method might also serve as a convenient procedure for the preparation of amino acids derivatives suitable for paper chromatography. Numerous references to the chromatography of dinitrophenylaminoacids have been recorded (*4, 5*).

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# Dynamic Control of Calcium, Phosphate, Citrate, and Glucose Levels in Blood Serum

## Effect of ACTH,* Adrenaline, Noradrenaline, Hydrocortisone, Parathormone, Insulin, and Glucagon

*Samuel Natelson, Joseph B. Pincus, and Gandolfo Rannazzisi*

**The effect of ACTH, adrenaline, noradrenaline, insulin, parathormone, hydrocortisone, and glucagon on the homeostatic mechanism controlling serum glucose, citrate, calcium, phosphorus, and potassium was studied in fasting rabbits and children. Citrate levels are kept in balance by ACTH, adrenaline, and glucagon, which raise the levels, and by insulin, which lowers them. Stress alone raises citrate levels, probably through adrenaline and ACTH effects.**

**Calcium levels in rabbits are markedly lowered by ACTH, given I.M., to tetany levels. Adrenaline has a similar, but mild, effect. I. M. parathormone raises calcium levels slowly. ACTH and parathormone may be the antagonists in keeping calcium levels constant.**

**Insulin, adrenaline, ACTH, glucagon, and parathormone lower phosphate levels. Major factors in lowering phosphate levels acutely evidently are insulin, which moves phosphate into the cell, and parathormone, which moves it through the kidney.**

**Insulin, adrenaline, ACTH, and glucagon lower potassium levels in the rabbit. Only noradrenaline causes a significant rise.**

**In the normal child only citrate moves in the same direction (elevates) as in the rabbit after ACTH.**

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*Since this paper was submitted the authors have ascertained that the substance responsible for lowering serum calcium levels in rabbits is distinct from ACTH. A fraction has been isolated from an ACTH preparation of porcine pituitaries free of ACTH activity (ascorbic acid depletion test) but very active in lowering calcium levels. The thyrotropic hormone, somatotrophin, prolactin, and oxytocin are without effect. Pitressin has a slight effect similar to adrenaline. Thus whenever ACTH is referred to in this paper we are referring to a mixture of ACTH and a rabbit calcium lowering factor.

**Children with CNS abnormality behaved like normal children except for 3 with febrile convulsions.**

**Homeostatic control of the constituents studied may be accomplished in the rabbit and the human by a balance of hormonal factors.**

ON THE BASIS OF OUR PREVIOUS STUDIES, it was concluded that glucose or insulin when injected in normal humans causes a fall in serum citrate levels (*27, 49*). When glucose was administered to a group of children who had been having repeated convulsive episodes, the citrate level did not fall. In many cases a rise of citrate occurred during the test (*51*). Adrenaline injected intramuscularly in normal humans resulted in a marked rise of citrate levels (*52*).

Other observers noted that normal individuals who responded with a fall of citrate levels when glucose was administered responded with a rise of serum citrate levels when the procedure was repeated after prolonged stress—10 days of starvation (*39*). This observation and our earlier ones suggested that the serum citrate level may be controlled by a homeostatic mechanism and that the response to stress influences this level.

Since calcium forms a chelate with citrate, a rise in citrate should cause a decrease in calcium ion concentration and may thus influence neuromuscular excitability (*43*). We therefore studied the effect of the "stress" hormones, adrenaline, ACTH, and cortisone, on the calcium, potassium, phosphate, citrate, and glucose levels in rabbits (*53*).

Intramuscular injection of adrenaline caused a rise in citrate levels. Also, a significant rise of serum citrate levels of the same order as obtained with adrenaline, but reaching its maximum later, was observed when ACTH was administered. We found that the potassium levels drop markedly on administration of adrenaline. This was confirmed by others(*16*). Calcium levels fell to as low as 50% of the initial value in rabbits when ACTH was administered. Convulsions ensued when the animals were mildly stimulated by smoke blown at their faces. This effect could be reproduced repeatedly. These measurements were taken during the first few hours after hormone administration. We therefore concluded that ACTH was probably involved in the acute homeostasis of serum calcium levels (*53*). That serum calcium levels are under acute homeostatic control is apparent from the constant serum levels observed in the normal human and the fact that when serum calcium levels are lowered by infusion of calcium-free serum, they return to normal rapidly (*29*).

The availability of a more accurate and precise method for assay of

calcium and potassium levels by X-ray spectrometry (*48*) which would insure the fact that our analyses would be specific for these elements and detect lesser changes with greater accuracy, and the availability of purer hormone preparations, prompted us to repeat some of our previous studies. We also extended the studies to include the effect of other hormones on the serum levels of calcium, potassium, phosphate, citric acid, and glucose during the period up to 5 hr. after injection.

## Procedure

Male rabbits weighing 3–4 kg. were used to make possible comparison of the results with those previously observed in female rabbits. Food, but not water, was withheld from the rabbits for 48 hr. before the test, to ensure stable initial values of the various blood constituents to be studied.

Care was taken not to alarm the animals during the experiment. The animals were rested in a quiet room for 24 hr. before the test. The ear vein was then slit and a first specimen obtained by discarding the first few drops and then collecting directly into acid-washed tubes. The blood flow was stopped with a pledget of cotton. Subsequent specimens were obtained by washing the initial cut gently with warm water to restart the blood flow. By using only water on the ear and drying before collecting, we prevented hemolysis and were able to obtain reliable potassium levels. We were able to obtain clear unhemolyzed serum even when collecting as many as seven specimens from the same site over a 5-hr. period. Washing the ear with alcohol or xylene promotes hemolysis and is irritating to the animal.

The hormones were injected intramuscularly or subcutaneously into the haunch of the animal. If more than 2 ml. was injected, the dose was divided between the two sides of the animal.

Calcium and potassium estimations were performed by X-ray spectrometry on 25-$\mu$l. samples run in triplicate (*48*). Citrate was estimated by a modification of a previously described method (*47*). Glucose levels were determined by a micro modification of the method of Folin and Wu (*22*). Phosphate was assayed by a micro modification of the method of Fiske and SubbaRow (*21*).

The hormones used were the purest that we could obtain for experimental purposes. ACTH purified on an oxycellulose column, obtained from Armour Pharmaceutical Company (Lot I-101), assayed 25 U. per milligram. This material was suspended in saline for intramuscular injection. In a few cases ACTH made by Wilson Laboratories (corticotropin) was used; it assayed 40 U. per milliliter.

*l*-Adrenaline and *l*-noradrenaline (*l*-arterenol) were used as recrystallized analytical-grade products and injected as aqueous solutions of the chlorides for adrenaline and as the tartrate for noradrenaline. Insulin was the commercially available regular insulin from Eli Lilly and Company, 40 U.S.P. U. per milliliter. Crystalline glucagon was obtained from Eli Lilly and Company and was suspended in saline for injection. Parathormone was obtained from Eli Lilly and Company and injected as a solution containing 100 U.S.P. U. per milliliter. Hydrocortisone alcohol was obtained from Eastman Kodak and Company and injected as a saline suspension. Cortef (The Upjohn Company; hydrocortisone alcohol, 50 mg. per milliliter of suspension) was also used in some cases.

Generally, the volume injected did not exceed 2 ml.; in the case of parathormone, however, the dose had to be divided and injected into both sides of the animal.

The human subjects studied were either normal children with no known hormonal disturbance who had been admitted to the hospital for some minor ailment or children who had been brought for treatment to the convulsion clinic at the Jewish Hospital of Brooklyn. They were hospitalized and started fasting in the morning of the day of testing. Blood was drawn from the vein after an electrocardiogram had been taken. Electrocardiograms were taken at hourly intervals thereafter before the blood was drawn.

## Results

The results listed in Table 1 confirm the earlier observations that adrenocorticotropic hormone will promptly lower calcium levels in the rabbit. This effect is obvious even at ½ hr., regardless of whether one uses oxycellulose-purified ACTH or the commercially available material (compare Rabbits 1–8 with Rabbits 9–11). In some instances, not shown in Table 1, in which we took 15-min. levels, the lowering in serum calcium was significant. Rabbits 5 and 9 had convulsions 1½ hr. after injection of the hormone in spite of the precautions we had taken not to excite the animals. Potassium and inorganic phosphate levels dropped significantly. Citrate levels rose markedly; in some cases (Rabbits 4 and 11) the rise exceeded 100%. Glucose levels were more variable. However, in every case a preliminary drop was followed by a significant rise.

A *t* test, comparing fasting values with maximums or minimums for

**Table 1.** Effect of Intramuscular ACTH* on Serum Ca, K, P, Citrate, and Glucose Levels† in Rabbits

| *Rabbit No.‡* | *Component* | *Fasting* | *Time after collection of specimen (hr.)* | | | | | |
|---|---|---|---|---|---|---|---|---|
| | | | ½ | 1 | 2 | 3 | 4 | 5 |
| | Ca | 11.69 | 10.45 | 10.30 | 8.39 | 8.00 | 8.84 | 10.07 |
| | K | 3.91 | 3.27 | 3.00 | 3.29 | 3.96 | 3.97 | 3.73 |
| 1 | P | 5.31 | 4.68 | 4.59 | 4.27 | 4.27 | 3.90 | 4.05 |
| | Citric | 6.08 | 6.23 | 7.30 | 6.95 | 7.51 | 7.37 | 7.15 |
| | Glucose | 124.5 | 133.2 | 91.3 | 121.0 | 103.2 | 141.1 | 131.3 |
| | Ca | 11.92 | 10.55 | 9.97 | 9.32 | 9.69 | 9.90 | 9.72 |
| | K | 3.90 | 2.86 | 3.02 | 2.79 | 3.52 | 3.01 | 2.84 |
| 2 | P | 6.16 | 5.32 | 5.45 | 5.12 | 6.00 | 5.91 | 5.91 |
| | Citric | 4.35 | 5.25 | 5.66 | 6.22 | 6.63 | 5.94 | 5.23 |
| | Glucose | 118.5 | 142.0 | 96.0 | 111.5 | 111.0 | 125.0 | 133.5 |
| | Ca | 11.47 | 9.32 | 8.70 | 8.05 | 9.92 | 11.0 | 10.56 |
| | K | 3.85 | 3.08 | 2.90 | 2.57 | 2.60 | 3.52 | 3.00 |
| 3 | P | 5.50 | 5.06 | 4.88 | 4.48 | 5.01 | 5.95 | 5.54 |
| | Citric | 4.45 | 4.57 | 5.16 | 4.37 | 5.61 | 4.85 | 4.89 |
| | Glucose | 110.0 | 129.5 | 113.5 | 121.0 | 148.5 | 147.0 | 109.0 |
| | Ca | 11.62 | 10.47 | 8.84 | 7.45 | 7.80 | 9.29 | 9.84 |
| | K | 3.78 | 3.24 | 3.01 | 3.22 | 3.10 | 3.42 | 3.20 |
| 4 | P | 4.12 | 3.82 | 3.82 | 4.78 | 3.48 | 3.00 | 3.34 |
| | Citric | 4.37 | 4.95 | 5.83 | 8.53 | 8.7 | 8.74 | 7.41 |
| | Glucose | 115.0 | 57.0 | 50.5 | 62.0 | 105.0 | 85.0 | 115.0 |
| | Ca | 11.93 | 11.22 | 10.82 | 8.99 | 6.62 | 5.90 | 6.01 |
| | K | 4.05 | 3.08 | 3.22 | 3.74 | 4.25 | 3.88 | 3.26 |
| 5 | P | 4.90 | 4.44 | 4.70 | 4.70 | 3.85 | 2.84 | 2.30 |
| | Citric | 5.66 | 7.18 | 7.42 | 7.46 | 8.15 | 9.19 | 11.05 |
| | Glucose | 115.0 | 65.0 | 69.5 | 113.0 | 98.0 | 130.0 | 146.5 |
| | Ca | 12.32 | 11.57 | 10.30 | 6.82 | 6.25 | 7.12 | 7.89 |
| | K | 3.42 | 2.92 | 3.13 | 3.46 | 3.36 | 3.37 | 3.68 |
| 6 | P | — | — | — | — | — | — | — |
| | Citric | 5.06 | 5.51 | 6.02 | 5.08 | 5.08 | 5.08 | 5.08 |
| | Glucose | 96.0 | 96.0 | 82.0 | 172.5 | 241.5 | 250.0 | — |
| | Ca | 12.01 | 11.96 | 10.93 | 7.91 | 7.18 | 8.92 | 10.05 |
| | K | 4.00 | 3.26 | 3.05 | 3.13 | 3.24 | 3.64 | 3.18 |
| 7 | P | 4.25 | 3.40 | 3.87 | 3.40 | 3.10 | 2.80 | 2.89 |
| | Citric | 5.20 | 5.62 | 6.86 | 7.32 | 7.92 | 7.86 | 6.90 |
| | Glucose | 134.0 | 89.5 | 85.5 | 125.5 | 118.5 | 110.5 | 133.0 |
| | Ca | 11.71 | 10.55 | 10.67 | 8.81 | 9.58 | 10.30 | 10.26 |
| | K | 4.48 | — | 4.68 | 4.16 | 4.03 | 3.44 | 3.80 |
| 8 | P | 4.75 | 5.23 | 4.40 | 4.35 | 3.55 | 3.18 | 3.55 |
| | Citric | 4.45 | 5.24 | 5.93 | 6.75 | 6.24 | 5.01 | 4.62 |
| | Glucose | 55.0 | 79.5 | 65.5 | 41.5 | 64.5 | 100.0 | 117.5 |

**(Continued)**

**Table 1. *(continued)***

| *Rabbit No.‡* | *Component* | *Fasting* | *Time after collection of specimen (hr.)* | | | | | |
|---|---|---|---|---|---|---|---|---|
| | | | ½ | 1 | 2 | 3 | 4 | 5 |
| | Ca | 13.18 | 13.08 | 12.11 | 8.58 | 8.00 | 8.09 | 8.93 |
| | K | 3.97 | 4.05 | 4.15 | 3.55 | 3.86 | 3.79 | 3.73 |
| 9 | P | 5.30 | 4.97 | 5.63 | 4.53 | 4.49 | 3.51 | 3.55 |
| | Citric | 6.33 | 9.38 | 10.02 | 9.70 | 9.80 | 10.52 | 11.1 |
| | Glucose | 86.4 | 75.0 | 77.7 | 115.8 | 123.0 | 159.8 | 151.1 |
| | Ca | 13.73 | 12.73 | 10.70 | 8.45 | 9.25 | 10.47 | 11.51 |
| | K | — | 3.90 | 3.28 | 4.15 | 3.95 | 3.66 | 3.95 |
| 10 | P | 4.88 | 4.32 | 5.84 | 5.16 | 4.32 | 4.44 | 4.48 |
| | Citric | 3.41 | 4.58 | 4.92 | 4.48 | 6.02 | 7.10 | 7.14 |
| | Glucose | 92.0 | 94.4 | 96.7 | 129.8 | 117.0 | 134.5 | 125.0 |
| | Ca | 11.72 | 10.24 | 10.65 | 9.62 | 9.15 | 9.55 | 9.77 |
| | K | 3.61 | 3.28 | 3.66 | 3.19 | 3.42 | 3.61 | 3.83 |
| 11 | P | 4.08 | 3.68 | 4.44 | 4.00 | 3.76 | 3.12 | 3.36 |
| | Citric | 3.37 | 5.35 | 6.26 | 6.41 | 7.76 | 6.23 | 6.15 |
| | Glucose | 151.5 | 139.1 | 117.3 | 111.8 | 115.0 | 140.3 | 154.0 |

*Dosage, 2 U./kg.

†In Tables 1–10, serum values are expressed in milligrams per 100 ml., except for K, which is given in milliequivalents per liter.

‡Rabbits 1–8 received purified ACTH, Rabbits 9–11, Wilson's corticotropin.

the various constituents (Table 1 and Fig. 1), showed that all were significant at the 1% level.

When ACTH was given in the same dosage (2 U. per kilogram) to normal children (Table 2), a "sawtooth" upward-moving curve was obtained; the maximum rise in calcium levels was shown to be significant by a *t* test. This was also true for the potassium levels, but the rise in potassium was barely significant at the 5% level. This was also true for phosphate levels, but the phosphate rise was delayed. Results obtained with glucose showed a significant minimum at the 5% level. Citrate levels rose consistently, except for Child 2, in whom a significant rise was not seen until the fifth hour. The mean data, as shown in Fig. 2, indicated a significant rise of citrate, on the basis of a *t* test. Only with respect to citrate did the children's response to ACTH resemble that of the rabbit.

In Table 3 are listed the results obtained on children with a CNS disturbance or hormone abnormality. None of the children studied, with the exception of Cases 7 and 8, had been subjected to repeated convulsive attacks, but Cases 4 and 5 had been reported to have had one convulsive episode each by their parents. Only Cases 2, 7, and 8

showed a response to ACTH different from that observed in the normal, the calcium level dropping significantly below the fasting level and remaining there. Case 7 was in difficulty and the test was abandoned at 4 hr. While Case 8 did not appear to be in trouble clinically, his electrocardiogram indicated, by a prolonged Q-T interval, that the 1-hr. and 2-hr. calcium levels would be low. Note that, in contrast to the rabbit, in Cases 2, 7, and 8 the serum citrate level dropped with the

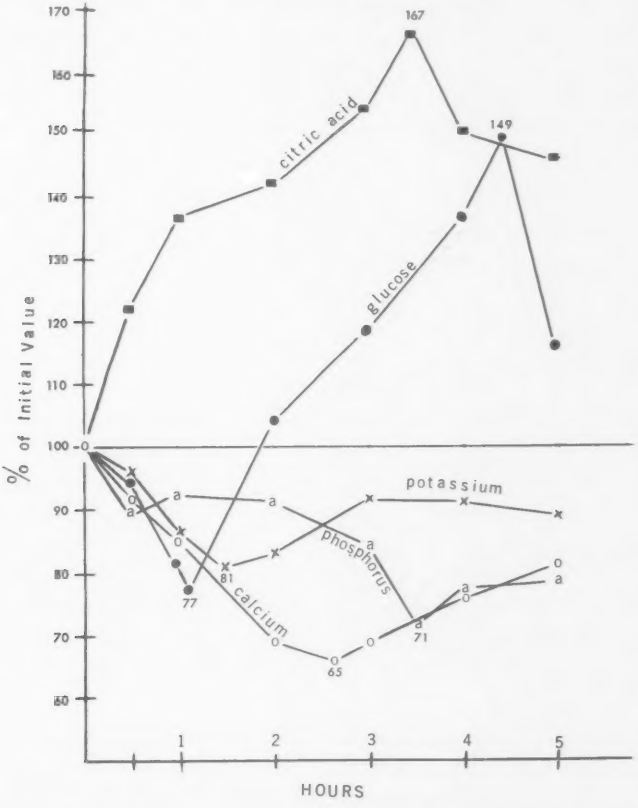


**Fig. 1.** Response of blood serum levels to 2 U./kg. of ACTH intramuscularly in fasting male rabbits weighing 3–4 kg. Mean values for 11 rabbits. In this and Fig. 2–8, time of occurrence of peak values was determined by averaging times of minimum and maximum values and averaging minimum or maximum values. Thus, extra point, calculated from mean maximum-change values obtained from tabular data, appears on each curve.

**Table 2.** Effect of Intramuscular ACTH* on Serum Ca, K, P, Citrate, and Glucose Levels in Normal Children

| *Child No.* | *Age (yr.) weight (kg.) and sex* | *Component* | *Fasting* | *Time after collection of specimen (hr.)* | | | | |
|---|---|---|---|---|---|---|---|---|
| | | | | *1* | *2* | *3* | *4* | *5* |
| | | Ca | 9.77 | 9.72 | 9.73 | 10.74 | 10.89 | 11.58 |
| | 2, | K | 4.20 | 4.24 | 4.03 | 5.03 | 4.97 | 4.94 |
| 1 | 15, | P | 4.70 | 4.79 | 4.40 | 4.52 | 4.97 | 5.37 |
| | M | Citric | 1.79 | 2.42 | 2.86 | 4.83 | 4.07 | 3.45 |
| | | Glucose | 83.1 | 98.3 | 88.5 | 73.4 | 82.0 | 74.5 |
| | | Ca | 10.33 | 10.52 | 10.88 | 11.09 | 10.17 | 11.12 |
| | 4, | K | 4.78 | 4.80 | 5.08 | 4.69 | — | 4.98 |
| 2 | 14, | P | 4.88 | 4.88 | 4.44 | 4.75 | 4.97 | 4.75 |
| | M | Citric | 3.27 | 3.27 | 3.10 | 3.36 | 3.20 | 3.66 |
| | | Glucose | 83.5 | 93.2 | 96.3 | 89.2 | 96.5 | 86.8 |
| | | Ca | 10.15 | 10.24 | 11.11 | 11.54 | 10.93 | 10.83 |
| | 2, | K | 4.46 | 4.33 | 4.73 | 4.43 | 4.17 | 4.34 |
| 3 | 13.3, | P | 4.91 | 5.12 | 5.20 | 5.12 | 5.58 | 5.49 |
| | M | Citric | 2.93 | 3.32 | 3.49 | 3.41 | 3.41 | 3.41 |
| | | Glucose | 75.6 | 81.8 | 61.3 | 88.9 | 81.8 | 81.8 |
| | | Ca | 10.20 | 10.58 | 10.80 | 10.19 | — | 10.90 |
| | 2, | K | 4.73 | 4.91 | 5.60 | 4.64 | — | 5.43 |
| 4 | 12.5, | P | 5.38 | 5.45 | 6.00 | 6.30 | — | 6.80 |
| | M | Citric | 2.05 | 2.25 | 2.27 | 2.25 | — | 2.25 |
| | | Glucose | 82.0 | 83.8 | 59.8 | 55.6 | — | 83.8 |
| | | Ca | 10.74 | 10.41 | 11.37 | 10.46 | 10.53 | 10.35 |
| | 2, | K | 4.98 | 4.60 | 4.46 | 4.62 | — | 4.34 |
| 5 | 15, | P | 4.92 | 5.15 | 5.42 | 5.45 | 6.15 | 5.92 |
| | M | Citric | 3.40 | 3.57 | 4.09 | 3.73 | 4.48 | 3.82 |
| | | Glucose | 70.4 | 89.2 | 68.6 | 63.30 | 43.7 | 68.6 |
| | | Ca | 9.54 | 10.40 | 9.56 | 11.64 | 11.48 | 10.81 |
| | 5, | K | — | 4.48 | 4.78 | 4.83 | 5.09 | 5.30 |
| 6 | 23, | P | 5.08 | 4.76 | 4.40 | 4.44 | 4.80 | 4.56 |
| | F | Citric | 2.97 | 3.41 | 3.53 | 4.14 | 4.27 | 4.07 |
| | | Glucose | 76.7 | 64.0 | 51.5 | 74.0 | 76.7 | 70.3 |

*Dosage, 2 U./kg.

drop in calcium level and then eventually rose to higher levels. The results in these cases cannot be pooled since each child is different. Case 4 had a milder condition, resembling those of Cases 7 and 8, but was able to raise his calcium level above the fasting level after a longer period.

Other results may be summarized as follows. Rabbits given adrenaline behave as though a small dose of ACTH had been administered,

except for the effect of adrenaline on glucose levels (Table 4, Fig. 3). This is consistent with the observation that adrenaline stimulates ACTH secretion in animals (*30, 33*).

Noradrenaline resembles adrenaline in its action but is generally milder in its effect (Table 5, Fig. 4). Its mild action on glucose levels is well documented (*4*). Its action on potassium is in the reverse direction from that of adrenaline, but its action on citrate, phosphorus, and calcium is in the same direction. Its action in slightly raising potassium levels in dogs has been noted by others (*46*).

Hydrocortisone causes a significant rise only in glucose levels, which continue to rise at 5 hr. (Table 6, Fig. 5). Note the similarity of the glucose curves for both cortisone and ACTH (Fig. 1 and 5). Effects on the other constituents were not significant on the basis of a *t* test, ex-

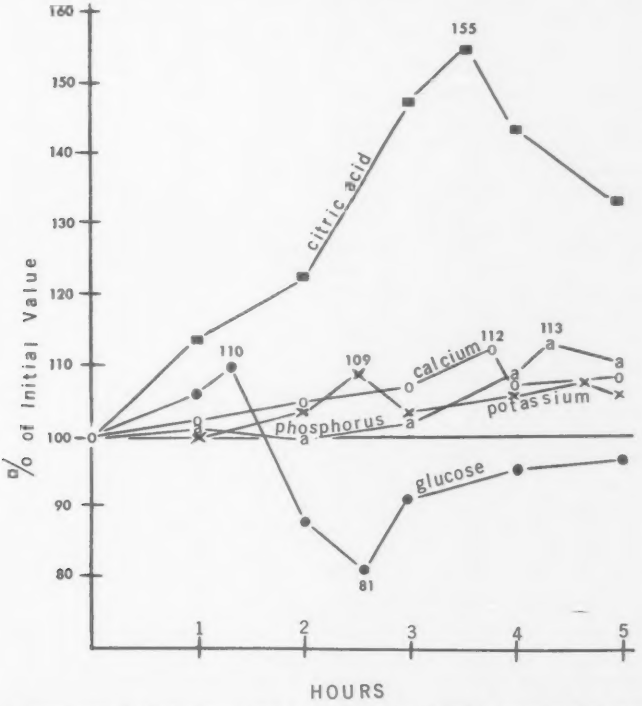


**Fig. 2.** Response of blood serum levels to 2 U./kg. of ACTH intramuscularly in normal children. Mean values for 6 children 2–5 years of age.

**Table 3.** Effect of Intramuscular ACTH* on Serum Ca, K, P, Citrate, and Glucose Levels in Children with Suspected CNS or Hormonal Aberration

| *Child No.†* | *Sex, age, weight diagnosis* | *Component* | *Fasting* | *Time after collection of specimen (hr.)* | | | | |
|---|---|---|---|---|---|---|---|---|
| | | | | *1* | *2* | *3* | *4* | *5* |
| | F, 15 mo., | Ca | 9.67 | 9.60 | 10.06 | 9.29 | 10.65 | 9.71 |
| | 11 kg., | K | 4.40 | — | 4.83 | 4.49 | 4.58 | 4.39 |
| 1 | paralysis of up- | P | 5.62 | 5.71 | 5.62 | 5.54 | 5.46 | 5.75 |
| | per and lower | Citric | 3.36 | 3.50 | 3.30 | 3.15 | 3.25 | — |
| | extremities | Glucose | 59.0 | 51.9 | 80.9 | 74.3 | 92.6 | 77.9 |
| | from birth | | | | | | | |
| | M, 6 yr., | Ca | 10.07 | 9.45 | 9.35 | 9.59 | 9.57 | 9.57 |
| | 21 kg., | K | 4.53 | 4.24 | 3.55 | 3.72 | 3.48 | 3.70 |
| 2 | after operation | P | 4.80 | 5.40 | 5.44 | 5.60 | 5.80 | 6.32 |
| | for hypospadias | Citric | 2.60 | 2.44 | 2.50 | 2.84 | 2.86 | 2.82 |
| | | Glucose | 60.0 | 104.4 | 96.0 | 87.0 | 96.0 | 103.2 |
| | M, 9 mo., | Ca | 9.51 | 9.86 | 9.36 | 10.23 | 10.00 | 9.26 |
| | 13 kg., after op- | K | 5.44 | — | — | 5.55 | 5.37 | — |
| 3 | eration for re- | P | 5.20 | 5.92 | 6.00 | 5.36 | 5.56 | 5.88 |
| | moval of sub- | Citric | 2.72 | 2.46 | 2.74 | 2.97 | 2.93 | — |
| | dural hematoma | Glucose | 65.0 | 70.7 | 64.0 | 67.0 | 62.7 | 62.7 |
| | M, 15 mo., | Ca | 10.02 | 9.22 | 9.24 | 9.88 | 9.85 | 10.47 |
| | 12 kg., 1 con- | K | 5.09 | 4.84 | 5.59 | 5.02 | 5.31 | 5.65 |
| 4 | vulsion of un- | P | 6.92 | 6.38 | 6.38 | 6.30 | 6.23 | 6.19 |
| | known origin | Citric | 2.97 | 2.75 | 2.81 | 2.56 | 2.48 | 1.60 |
| | | Glucose | 46.5 | 60.3 | 72.1 | 79.3 | 61.6 | 72.7 |
| | F, 20 mo., | Ca | 9.51 | 9.58 | 9.75 | 10.07 | 9.96 | 10.51 |
| | 11 kg., hemi- | K | 4.04 | 4.19 | 4.28 | 4.28 | 4.32 | 4.49 |
| 5 | plegia after sin- | P | 5.15 | 5.31 | 5.50 | 5.77 | 5.57 | 5.38 |
| | gle convulsion | Citric | 2.32 | 2.28 | 2.28 | 2.49 | 2.52 | 2.86 |
| | of unknown ori- | Glucose | 56.0 | 54.5 | 51.2 | 63.0 | 62.0 | 61.6 |
| | gin | | | | | | | |
| | F, 20 mo., | Ca | 9.98 | 9.79 | 9.77 | 9.61 | 9.78 | 9.27 |
| | 17 kg., | K | 4.75 | 3.54 | 3.56 | — | 3.75 | 4.00 |
| 6 | convulsions of | P | 4.67 | 4.49 | 4.71 | 5.29 | 4.93 | 4.67 |
| | unknown origin | Citric | 2.66 | 2.70 | 2.47 | 2.57 | 2.73 | 2.73 |
| | | Glucose | 58.2 | 99.0 | 78.8 | 62.8 | 60.0 | 27.3 |
| | F, 13 mo., | Ca | 9.15 | 2.96 | 4.71 | 4.41 | 5.70 | — |
| | 10 kg., repeated | K | 3.95 | 3.97 | 4.66 | 5.15 | 5.56 | — |
| 7 | febrile convul- | P | 4.71 | 4.44 | 4.63 | 5.10 | 5.65 | — |
| | sions, afebrile | Citric | 1.90 | 1.54 | 1.90 | 1.87 | 2.14 | — |
| | at time of test | Glucose | 59.0 | 80.0 | 74.5 | 72.0 | 65.8 | — |
| | F, 4 yr., | Ca | 9.30 | 9.02 | 8.88 | 9.61 | 9.89 | 9.26 |
| | 20 kg., | K | 4.68 | 4.62 | 4.69 | 5.76 | 5.63 | 5.33 |
| 8 | several febrile | P | 4.78 | 4.97 | 5.0 | 5.33 | 5.54 | 5.54 |
| | convulsions | Citric | 2.52 | 2.40 | 2.45 | 2.77 | 3.16 | 3.06 |
| | | Glucose | 66.5 | 72.5 | 75.0 | 78.0 | 94.0 | 80.0 |

*ACTH dosage, 2 U./kg.

†Cases 1–4 received purified ACTH, Cases 5–8, Wilson's corticotropin.

**Table 4.** Effect of Intramuscular Adrenaline* on Serum Ca, K, P, Citrate, and Glucose Levels in Rabbits

| *Rabbit No.* | *Component* | *Fasting* | *Time after collection of specimen (hr.)* ½ | 1 | 1½ | 2 | 3 |
|---|---|---|---|---|---|---|---|
| | Ca | 13.04 | 12.77 | 12.72 | 12.58 | 12.46 | 12.27 |
| | K | 3.05 | 3.65† | 3.78† | 3.98† | 2.72 | 2.92 |
| 1 | P | 3.48 | 3.15 | 3.92 | 2.78 | 2.52 | 2.40 |
| | Citric | 4.52 | 6.59 | 5.65 | 5.84 | 4.64 | 5.02 |
| | Glucose | 120.9 | 166.9 | 203.6 | 235.1 | 258.1 | 329.2 |
| | Ca | 11.51 | 11.69 | 11.05 | 12.18 | 10.73 | 11.42 |
| | K | 4.10 | 3.34 | 3.33 | 3.76 | 4.15 | 4.32 |
| 2 | P | 4.82 | 4.04 | 3.80 | 3.88 | 3.84 | 3.49 |
| | Citric | 5.95 | 6.86 | 7.25 | 6.80 | 6.53 | 4.57 |
| | Glucose | 104.4 | 177.3 | 283.6 | 324.8 | 368.71 | 374.0 |
| | Ca | 13.18 | 12.22 | 12.71 | 12.75 | 12.59 | 12.10 |
| | K | 4.00 | 3.33 | 3.30 | 3.54 | 3.75 | 3.78 |
| 3 | P | 5.07 | 3.17 | 2.90 | 3.09 | 2.86 | 2.98 |
| | Citric | 6.02 | 6.15 | 6.02 | 5.88 | 5.62 | 5.16 |
| | Glucose | 78.8 | 156.2 | 204.9 | 255.7 | 262.2 | 252.6 |
| | Ca | 13.74 | 13.25 | 13.27 | 12.86 | 12.99 | 12.46 |
| | K | 3.42 | 3.05 | 3.25 | 3.14 | 3.13 | 3.25 |
| 4 | P | 6.60 | 5.22 | 5.70 | 4.95 | 5.22 | 4.96 |
| | Citric | 4.78 | 6.59 | 6.32 | 5.43 | 5.21 | 4.93 |
| | Glucose | 108.4 | 229.4 | 266.0 | 320.5 | 356.6 | 375.9 |
| | Ca | 12.64 | 12.63 | 12.73 | 12.17 | 12.55 | 11.94 |
| | K | 3.17 | 3.00 | 3.02 | 3.14 | 3.45 | 3.20 |
| 5 | P | 3.16 | 2.83 | 2.91 | 3.12 | 3.25 | 3.20 |
| | Citric | 3.00 | 3.26 | 2.93 | 2.64 | 2.24 | 2.46 |
| | Glucose | 115.5 | 198.5 | 247.0 | 284.0 | 323.5 | 365.0 |
| | Ca | 11.92 | 12.58 | 12.72 | 12.79 | 11.40 | 12.34 |
| | K | 3.17 | 3.09 | 2.92 | 2.93 | 2.76 | 2.89 |
| 6 | P | 4.08 | 3.75 | 3.59 | 3.34 | 3.06 | 3.02 |
| | Citric | 7.72 | 7.45 | 6.25 | 6.18 | 6.65 | 6.54 |
| | Glucose | 101.5 | 210.0 | 258.0 | 299.0 | 286.0 | 281.0 |
| | Ca | 12.10 | 11.50 | 11.43 | 11.30 | 10.49 | 10.57 |
| | K | 4.11 | 2.48 | 2.94 | 3.02 | 3.21 | 2.92 |
| 7 | P | 3.29 | 2.58 | 2.33 | 2.37 | 3.00 | 3.62 |
| | Citric | 7.18 | 7.48 | 7.14 | 6.62 | 5.74 | 6.63 |
| | Glucose | 79.5 | 210.1 | 282.0 | 222.0 | 358.2 | 353.0 |
| | Ca | 11.47 | 11.62 | 11.18 | 11.33 | 11.11 | 10.72 |
| | K | 3.57 | 3.05 | 2.97 | 2.87 | 3.26 | 3.28 |
| 8 | P | 3.64 | 2.53 | 2.70 | 2.26 | 2.61 | 2.80 |
| | Citric | 5.86 | 6.96 | 6.59 | 5.86 | 5.57 | 4.87 |
| | Glucose | 105.5 | 160.0 | 180.0 | 222.5 | 257.0 | 201.0 |

(Continued)

**Table 4. *(continued)***

| *Rabbit No.* | *Component* | *Fasting* | *Time after collection of specimen (hr.)* ½ | 1 | 1½ | 2 | 3 |
|---|---|---|---|---|---|---|---|
| | Ca | 12.20 | 12.35 | 11.45 | 11.87 | 11.73 | 11.37 |
| | K | 3.37 | 2.90 | 2.65 | 2.70 | 2.43 | 2.70 |
| 9 | P | 5.30 | 5.16 | 4.82 | 4.52 | 4.60 | 4.21 |
| | Citric | 3.76 | 4.85 | 4.71 | 4.75 | 4.78 | 4.89 |
| | Glucose | 90.5 | 219.6 | 286.4 | 326.9 | 292.0 | 296.8 |
| | Ca | 11.67 | 11.29 | 11.33 | 11.23 | 11.12 | 11.54 |
| | K | 3.05 | 2.70 | 2.49 | 2.05 | 2.41 | 3.03 |
| 10 | P | 5.06 | 4.17 | 3.91 | 4.00 | 3.91 | 4.05 |
| | Citric | 2.77 | 3.30 | 3.34 | 3.20 | 2.80 | 2.86 |
| | Glucose | 120.8 | 189.1 | 242.6 | 290.4 | 298.1 | 336.7 |

*Dosage, 0.1 mg./kg.
†Slight hemolysis.

cept that the early rise in citrate level was barely significant at the 5% level.

Insulin causes significant depression of potassium, citrate, phosphorus, and glucose levels (Table 7, Fig. 6). The depression in potassium levels (*3, 42, 65*) and phosphate levels (*60, 66*) has been observed repeatedly by others in man and various animals. While a depression of serum calcium level was observed in each case, a *t* test indicated that the results were not quite significant at the 5% level. If more rabbits are studied, this depression may be found to be significant.

Glucagon's effect is extremely rapid and of short duration (Table 8, Fig. 7). The maximum changes are all significant, although the changes in calcium levels are significant only at the 5% level. Note that the changes in potassium levels are delayed after the immediate effect of glucagon on glucose, citrate, and phosphate levels have ended. The drop in phosphate with glucagon is well documented in the literature in man and dogs (*32, 67*).

The effect of parathormone on the elevation of calcium is significant (Table 9, Fig. 8). The calcium level begins to decline at the end of the fourth hour. A transient rise in citrate level is noted followed by a fall below the fasting level. The phosphate depression continues to the third hour. The effect on glucose is not significant, the values oscillating as they do in the fasting animal. Potassium values also oscillate except for a transient rise at the beginning, at the time that the phosphate level shows a minimum.

Table 10 illustrates the results obtained in 2 normal children after

adrenaline administration. Statistical tests cannot be reasonably applied to so small a number, but the rise in glucose, drop in potassium and phosphate, and rise in citrate are observed in both cases. In both subjects small changes in calcium were observed, a minimum being seen as in the rabbits.

## Discussion

The objective of this study was to investigate the acute effects of certain hormones on serum calcium and potassium levels and other

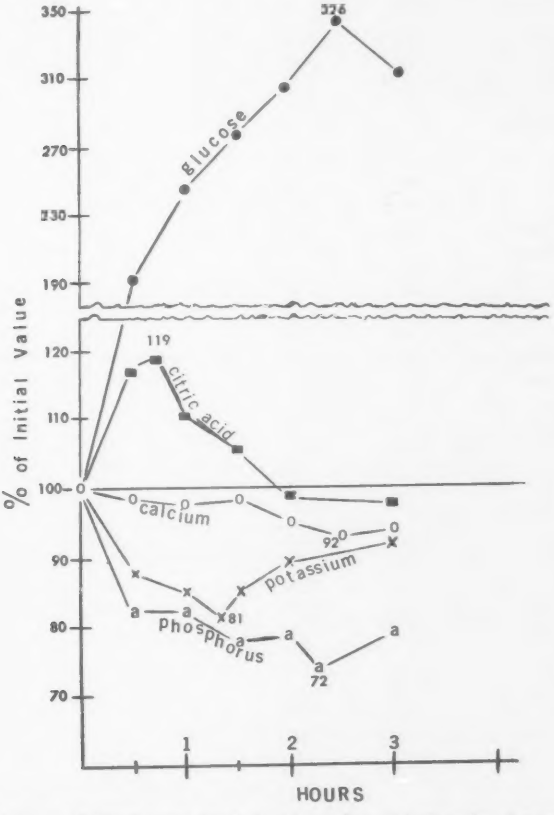


**Fig. 3.** Response of blood serum levels to 0.1 mg./kg. of *l*-adrenaline intramuscularly in fasting male rabbits. Mean values for 10 rabbits.

**Table 5.** Effect of Intramuscular *l*-Noradrenaline* on Serum Ca, K, P, Citrate, and Glucose Levels in Rabbits

| *Rabbit No.* | *Component* | *Fasting* | *Time after collection of specimen (hr.)* ½ | *1* | *1½* | *2* | *3* |
|---|---|---|---|---|---|---|---|
| | Ca | 13.60 | 13.33 | 12.92 | 12.89 | 12.54 | 12.58 |
| | K | 3.55 | 4.68 | 4.02 | 4.52 | 4.22 | 4.39 |
| 1 | P | 4.32 | 4.69 | 4.53 | 4.57 | 4.08 | 4.32 |
| | Citric | 5.1 | 5.63 | 5.56 | 5.3 | 5.43 | 5.1 |
| | Glucose | 109.2 | 108.5 | 107.0 | 126.1 | 123.0 | 129.9 |
| | Ca | 12.73 | 13.05 | 12.72 | 12.0 | 12.14 | 12.16 |
| | K | 3.12 | 3.82 | 3.68 | 3.10 | 3.19 | 3.64 |
| 2 | P | 6.23 | 4.74 | 4.88 | 3.9 | 3.34 | 3.53 |
| | Citric | 4.71 | 5.84 | 5.84 | 6.51 | 6.08 | 6.41 |
| | Glucose | 133.7 | 117.9 | 122.7 | 92.7 | 95.9 | 66.8 |
| | Ca | 12.92 | 13.27 | 12.94 | 12.41 | 12.66 | 12.57 |
| | K | 3.93 | 4.22 | 3.93 | 3.92 | 4.03 | 4.19 |
| 3 | P | 4.88 | 4.7 | 4.08 | 4.57 | 4.26 | 4.8 |
| | Citric | 4.93 | 5.2 | 4.81 | 4.89 | 5.06 | 6.44 |
| | Glucose | 71.5 | 96.3 | 54.8 | 78.4 | 75.5 | 107.6 |
| | Ca | 12.72 | 12.83 | 12.87 | 12.78 | 13.0 | 12.66 |
| | K | 4.18 | 4.95 | 4.44 | 4.32 | 4.32 | 3.96 |
| 4 | P | 4.1 | 3.96 | 4.10 | 4.15 | 4.4 | 4.45 |
| | Citric | 6.19 | 6.68 | 7.42 | 7.8 | 8.43 | 7.8 |
| | Glucose | 96.9 | 121.9 | 125.3 | 102.0 | 96.0 | 118.6 |
| | Ca | 14.19 | 13.16 | 13.8 | 13.71 | 13.16 | 13.88 |
| | K | 3.20 | 3.81 | 4.01 | 3.98 | 3.81 | 4.03 |
| 5 | P | 5.95 | 5.37 | 5.42 | 5.46 | 5.86 | 5.86 |
| | Citric | 6.08 | 7.17 | 7.1 | 6.86 | 6.63 | 6.69 |
| | Glucose | 84.0 | 114.6 | 64.9 | 46.0 | 77.2 | 61.8 |
| | Ca | 13.31 | 13.05 | 12.87 | 12.94 | 12.88 | 12.78 |
| | K | 4.14 | 4.34 | 4.10 | 4.04 | 4.41 | 4.53 |
| 6 | P | 4.70 | 4.20 | 4.18 | 4.10 | 4.10 | 4.07 |
| | Citric | 8.52 | 9.18 | 9.42 | 8.62 | 7.68 | 9.35 |
| | Glucose | 116.5 | 133.2 | 126.6 | 128.9 | 123.6 | 128.9 |
| | Ca | 13.37 | 12.73 | 13.36 | 13.91 | 12.90 | 14.73 |
| | K | 4.04 | 4.01 | 4.29 | 3.87 | 3.77 | 3.81 |
| 7 | P | 3.95 | 3.64 | 3.68 | 3.77 | 3.95 | 3.86 |
| | Citric | 6.10 | 6.02 | 5.74 | 5.61 | 5.7 | 5.52 |
| | Glucose | 128.4 | 123.0 | 112.3 | 133.7 | 121.4 | 126.2 |

*Dosage, 0.1 mg./kg.

components which directly or indirectly affect the activity of these ions. We were primarily interested in probing the mechanism which maintains these ions at constant levels in the living organism. This is distinct from the long-range effects, which are secondary to complex changes in the body.

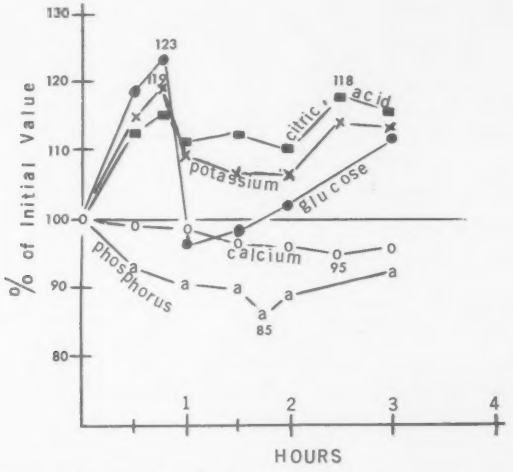


**Fig. 4.** Response of blood serum levels to 0.1 mg./kg. of *l*-noradrenaline intramuscularly in fasting male rabbits. Mean values for 7 rabbits.

### Glucose

Adrenaline, insulin, and glucagon produce immediate and rapid changes in glucose levels (Fig. 3, 6, and 7). The effect of glucagon in raising blood sugar levels is of much shorter duration than that of adrenaline although the action of these two hormones seems to be similar (*36, 41*).

The effect of noradrenaline in raising glucose levels is much less than that of adrenaline (Fig. 4). This is true both in rabbits (*7*) and in humans (*4, 28, 62*). The adrenal medulla secretes large amounts of adrenaline when stimulated by insulin hypoglycemia but very small amounts of noradrenaline (*9*). These observations seem to indicate that adrenaline but not noradrenaline is significant in acutely raising serum glucose levels when required.

The shapes of the curves for glucose resemble each other when ACTH and cortisone are administered (Fig. 1 and 5). The fact that

**Table 6.** Effect of Intramuscular Hydrocortisone Alcohol on Serum Ca, K, P, Citrate, and Glucose Levels in Rabbits

| *Rabbit No.** | *Component* | *Fasting* | *Time after collection of specimen (hr.)* | | | | | |
|---|---|---|---|---|---|---|---|---|
| | | | ½ | 1 | 2 | 3 | 4 | 5 |
| | Ca | 13.15 | 12.82 | 12.69 | 13.17 | 12.83 | 13.02 | 11.94 |
| | K | 3.01 | 2.70 | 2.81 | 2.88 | 2.85 | 3.03 | 3.02 |
| 1 | P | 3.66 | 3.40 | 4.21 | 3.61 | 4.08 | 3.36 | 3.83 |
| | Citric | 4.67 | 4.92 | 5.47 | 5.76 | 6.04 | 6.62 | 6.26 |
| | Glucose | 102.2 | 78.7 | 67.5 | 129.7 | 134.0 | 131.2 | 140.4 |
| | Ca | 13.40 | 12.92 | 13.04 | 12.84 | 13.0 | 11.78 | 12.60 |
| | K | 3.16 | 3.36 | 3.34 | 3.11 | 3.12 | — | 3.25 |
| 2 | P | 5.79 | 6.45 | 6.52 | 5.70 | 5.35 | 3.88 | 4.03 |
| | Citric | 4.29 | 4.14 | 4.07 | 4.33 | 4.56 | 4.71 | 4.52 |
| | Glucose | 176.5 | 142.5 | 147.5 | 178.0 | 175.5 | 202.0 | 223.5 |
| | Ca | 12.38 | 12.02 | 12.23 | 12.65 | 12.94 | 12.82 | 12.59 |
| | K | 3.10 | 3.55 | 3.32 | 3.38 | 3.44 | 3.93 | 3.70 |
| 3 | P | 4.80 | 4.42 | 4.27 | 3.92 | 4.30 | 4.11 | 4.23 |
| | Citric | 8.5 | 9.61 | 8.94 | 7.76 | 6.34 | 5.4 | 4.89 |
| | Glucose | 135.1 | 106.9 | 101.6 | 158.7 | 201.7 | 236.4 | 239.3 |
| | Ca | 12.86 | 13.15 | 13.33 | 13.40 | 13.26 | 13.00 | 13.06 |
| | K | 3.35 | 3.46 | 3.64 | 3.67 | 3.77 | 3.77 | 3.39 |
| 4 | P | 6.01 | 5.43 | 4.94 | 5.04 | 5.23 | 5.19 | 5.27 |
| | Citric | 5.37 | 5.80 | 5.90 | 4.94 | 4.94 | 4.54 | 4.94 |
| | Glucose | 153.0 | 122.0 | 115.0 | 111.0 | 145.0 | 161.5 | 220.0 |
| | Ca | 11.83 | 12.81 | 12.02 | 11.61 | 12.60 | 11.63 | 12.10 |
| | K | 2.94 | 3.12 | 3.57 | 2.97 | 3.29 | — | 3.34 |
| 5 | P | 3.66 | 3.66 | 4.08 | 4.34 | 4.47 | 4.34 | 4.51 |
| | Citric | 8.29 | 6.42 | 8.46 | 8.29 | 7.14 | 7.94 | 7.66 |
| | Glucose | 163.5 | 166.6 | 183.8 | 188.8 | 198.2 | 230.4 | 230.4 |
| | Ca | 12.58 | 12.68 | 12.56 | 12.49 | 13.0 | 13.07 | 13.12 |
| | K | 3.04 | 3.18 | — | 3.07 | — | 3.00 | 3.10 |
| 6 | P | 3.94 | 4.08 | 4.08 | 4.34 | 4.64 | 4.50 | 4.57 |
| | Citric | 2.65 | 3.15 | 3.15 | 2.57 | 2.07 | 2.14 | 2.14 |
| | Glucose | 132.0 | 145.0 | 141.0 | 155.0 | 163.5 | 176.0 | 194.0 |
| | Ca | 13.84 | 14.74 | 14.59 | 14.43 | 14.42 | 14.17 | 14.25 |
| | K | 2.90 | — | 3.21 | 3.27 | 3.21 | 3.50 | 3.58 |
| 7 | P | 4.82 | 4.34 | 4.43 | 4.30 | 4.34 | 4.74 | 4.69 |
| | Citric | 5.22 | 5.15 | 5.15 | 5.51 | 5.65 | 5.54 | 5.73 |
| | Glucose | 135.5 | 144.5 | 121.5 | 172.5 | 200.0 | 245.0 | 254.0 |

*Dosage: Rabbits 1–3, 6 mg./kg.; Rabbits 4–7, 20 mg./kg.

the serum glucose level continues to rise after the curve obtained with ACTH has begun to recede, suggests that in both these cases this is a steroid hormone effect. With ACTH, endogenous corticosterone secretion probably produces this effect, limited by the amount of steroid

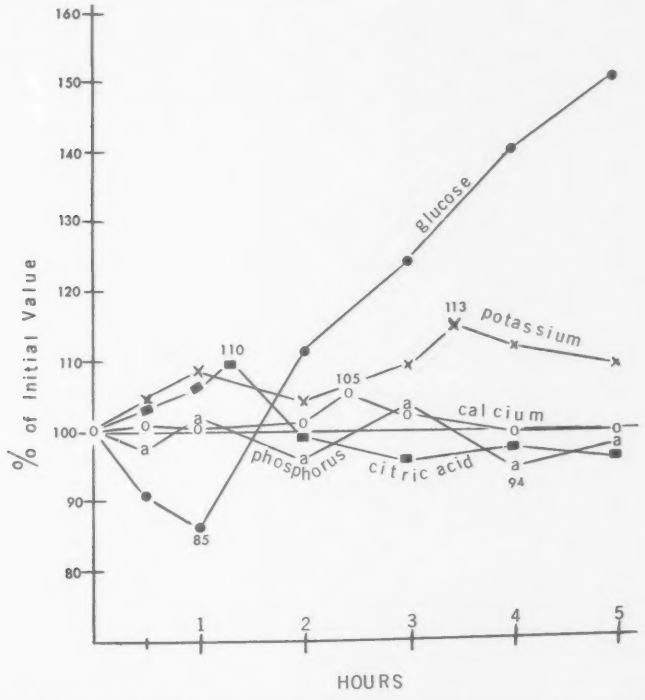


**Fig. 5.** Response of blood serum levels to 20 mg./kg. of hydrocortisone alcohol intramuscularly in fasting male rabbits. Mean values for 7 rabbits.

hormone the animal produces under the stimulus. With cortisone the amount of steroid hormone administered was much larger than the animal could have produced during the experiment. In any case, only adrenaline and glucagon are antagonistic to insulin in rapid action on serum glucose levels and not hydrocortisone or ACTH (*8, 57, 64*).

From these observations we conclude that glucagon and adrenaline are the immediate factors responsible for raising glucose levels and that insulin is the counterbalancing force.

**Table 7.** Effect of Subcutaneous Insulin* on Serum Ca, K, P, Citrate, and Glucose Levels in Rabbits

| *Rabbit No.* | *Component* | *Fasting* | *Time after collection of specimen (hr.)* | | | |
|---|---|---|---|---|---|---|
| | | | ½ | 1 | 2 | 3 |
| | Ca | 13.94 | 13.78 | 12.72 | 13.61 | 13.85 |
| | K | 4.76 | — | — | 3.80 | 4.35 |
| 1 | P | 3.76 | 3.76 | 3.19 | 3.92 | 4.07 |
| | Citric | 5.17 | 4.65 | 4.42 | 3.97 | 3.97 |
| | Glucose | 86.2 | 12.4 | 11.8 | 8.25 | 4.7 |
| | Ca | 12.85 | 13.10 | 13.16 | 12.65 | 12.84 |
| | K | 3.62 | 3.55 | 3.45 | 3.53 | 3.85 |
| 2 | P | 3.33 | 3.12 | 2.96 | 3.00 | 3.38 |
| | Citric | 5.44 | 5.07 | 5.07 | 4.54 | 4.79 |
| | Glucose | 150.0 | 124.5 | 108.5 | 85.0 | 121.2 |
| | Ca | 12.40 | 12.37 | 13.00 | 11.88 | 12.63 |
| | K | 3.57 | 3.54 | 3.32 | 3.40 | 3.62 |
| 3 | P | 4.15 | 3.92 | 3.23 | 3.42 | 3.54 |
| | Citric | 4.93 | 5.0 | 4.93 | 4.71 | 4.67 |
| | Glucose | 183.0 | 111.5 | 60.5 | 51.7 | 54.6 |
| | Ca | 14.41 | 15.52 | 14.91 | 12.87 | 12.87 |
| | K | 4.21 | 4.11 | 4.16 | 3.44 | 3.47 |
| 4 | P | 5.65 | 5.61 | 3.80 | 3.07 | 3.53 |
| | Citric | 5.28 | 5.60 | 3.33 | 4.75 | 4.75 |
| | Glucose | 162.0 | 111.2 | 73.5 | 44.2 | 45.4 |
| | Ca | 13.27 | 13.57 | 12.77 | 12.70 | 12.53 |
| | K | 4.33 | 3.54 | 3.59 | 3.33 | 3.78 |
| 5 | P | 3.88 | 2.95 | 2.13 | 2.42 | 2.92 |
| | Citric | 6.11 | 5.58 | 5.14 | 4.87 | 4.99 |
| | Glucose | 178.0 | 78.4 | 39.5 | 27.7 | 32.4 |

*Dosage, 0.25 U./kg.

## Citrate

Serum citrate levels are markedly elevated by adrenaline and ACTH (Fig. 1 and 3) and depressed when glucose levels are dropping due to the action of insulin (Fig. 6). Insulin also drops citrate levels in humans (*27*). Thus, movement of glucose into the serum is accompanied by a rise in citrate. This is also true for glucagon (Fig. 7) as well as adrenaline (Fig. 3). In this respect glucagon and adrenaline are antagonistic to the action of insulin.

On inspection of the adrenaline curve (Fig. 6), one notes that there is an initial rapid rise in citrate, paralleling the rise in glucose. As the glucose level continues to rise, the citrate level begins to fall. This sug-

gests that this is an insulin effect. The elevated blood glucose stimulates secretion of insulin, which tends to drive the citrate levels down as the glucose moves back into the tissues.

Examination of the curves for ACTH in rabbits (Fig. 1) and chil-

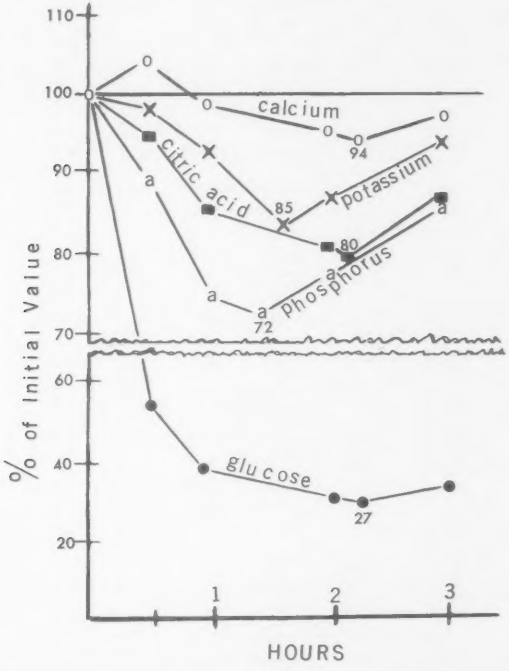


**Fig. 6.** Response of blood serum levels to 0.25 U./kg. of insulin subcutaneously in fasting male rabbits. Mean values for 5 rabbits. Vertical scale has been expanded, because effects observed were uniformly smaller.

dren (Fig. 2) shows a marked rise in citric acid serum levels in both cases. The question arises as to what is the prime cause of the rise of serum citrate levels: adrenaline or ACTH? We observed that stress of any form in a rabbit will result in a prompt rise in citrate levels. The fasting citrate levels for rabbits (Table 1) range from a low of 3.37 to a high of 6.33. Only 2 animals, Rabbits 1 and 9, had levels above 6 mg./100 ml. Both of these animals were very apprehensive at the start of the test.

**Table 8.** Effect of Intramuscular Crystalline Glucagon* on Serum Ca, K, P, Citrate, Glucose Levels in Rabbits

| *Rabbit No.* | *Component* | *Fasting* | *Time after collection of specimen (hr.)* | | | | | | |
|---|---|---|---|---|---|---|---|---|---|
| | | | ¼ | ¾ | 1¼ | 2 | 3 | 4 | 5 |
| | Ca | 11.70 | 11.85 | 11.97 | 10.77 | 10.76 | 10.11 | 11.08 | 10.58 |
| | K | 4.00 | 4.13 | 4.18 | 4.00 | 4.04 | 3.68 | 3.80 | 4.13 |
| 1 | P | 5.61 | 4.87 | 3.71 | 4.08 | 4.08 | 4.32 | 4.52 | 4.84 |
| | Citric | 4.06 | 5.10 | 6.00 | 6.00 | 4.74 | 4.12 | 4.06 | 4.36 |
| | Glucose | 77.2 | 154.0 | 126.0 | 131.8 | 95.7 | 103.2 | 80.5 | 116.7 |
| | Ca | 13.65 | 13.38 | 13.02 | 14.28 | 13.22 | 13.33 | 13.84 | 14.0 |
| | K | 3.44 | 3.57 | 3.39 | 3.75 | 3.54 | 4.08 | 4.52 | 4.94 |
| 2 | P | 4.59 | 4.60 | 4.25 | 4.34 | 4.25 | 5.02 | 4.21 | 4.25 |
| | Citric | 4.26 | 5.26 | 4.88 | 4.58 | 4.51 | 4.13 | 4.36 | 3.76 |
| | Glucose | 96.6 | 138.0 | 147.5 | 155.8 | 135.7 | 157.5 | 118.0 | 122.7 |
| | Ca | 14.45 | 14.02 | 13.98 | 14.44 | 13.90 | 14.30 | 13.45 | 13.87 |
| | K | 3.67 | 3.42 | 3.32 | 3.10 | 3.53 | 3.64 | 3.37 | 3.82 |
| 3 | P | 4.90 | 4.94 | 4.53 | 4.61 | 4.98 | 4.78 | 4.78 | 5.06 |
| | Citric | 4.19 | 4.08 | 3.96 | 3.56 | 3.56 | 2.59 | 2.96 | 3.11 |
| | Glucose | 82.0 | 106.2 | 82.0 | 82.0 | 84.9 | 106.2 | 110.5 | 109.2 |
| | Ca | 13.76 | 12.44 | 11.92 | 13.27 | 13.35 | 12.81 | 12.47 | 12.46 |
| | K | 4.83 | 4.80 | 3.86 | 4.30 | — | 3.78 | 3.34 | 3.90 |
| 4 | P | 3.79 | 4.12 | 3.44 | 3.36 | 3.86 | 3.56 | 3.61 | 3.73 |
| | Citric | 5.5 | 6.15 | 7.27 | 6.29 | 5.14 | 4.07 | 4.41 | 4.63 |
| | Glucose | 98.5 | 135.6 | 96.0 | 99.8 | 81.5 | 94.8 | 93.0 | 109.4 |
| | Ca | 13.90 | 14.48 | 14.01 | 12.67 | 12.30 | 11.76 | 12.47 | 12.71 |
| | K | 4.13 | 4.53 | 4.0 | 3.69 | 3.83 | 3.55 | 3.92 | 3.82 |
| 5 | P | 5.0 | 3.68 | 2.92 | 3.44 | 4.08 | 4.00 | 4.56 | 4.64 |
| | Citric | 7.14 | 7.97 | 7.81 | 7.52 | 7.52 | 7.44 | 7.40 | 7.10 |
| | Glucose | 168.0 | 207.8 | 162.0 | 133.3 | 118.0 | 95.5 | 118.0 | 139.8 |
| | Ca | 11.96 | 11.83 | 11.78 | 11.26 | 10.81 | 11.13 | 11.95 | 11.96 |
| | K | 3.93 | 3.71 | 3.52 | 3.72 | 3.55 | 3.33 | 3.51 | 3.63 |
| 6 | P | 4.16 | 3.82 | 3.82 | 4.04 | 4.08 | 4.0 | 3.56 | 3.82 |
| | Citric | 4.85 | 5.14 | 4.88 | 4.48 | 4.27 | 3.90 | 3.0 | 2.85 |
| | Glucose | 87.3 | 139.2 | 87.3 | 76.8 | 50.7 | 98.0 | 89.1 | 111.5 |
| | Ca | 12.25 | 11.42 | 11.19 | 10.81 | 11.64 | 11.61 | 12.15 | 11.67 |
| | K | 3.88 | 3.92 | 3.59 | 3.79 | 3.79 | 3.72 | 3.79 | 4.12 |
| 7 | P | 4.24 | 3.36 | 2.80 | 3.12 | 3.36 | 3.44 | 3.44 | 3.84 |
| | Citric | 4.65 | 5.67 | 5.78 | 4.97 | 4.71 | 4.77 | 4.30 | 4.11 |
| | Glucose | 110.5 | 149.5 | 126.6 | 120.6 | 92.0 | 82.0 | 93.6 | 79.1 |

*Dosage, 0.14 mg./kg.

To study the effect of stress on citrate levels, we stimulated rabbits by dropping xylene into their eyes. This caused them to scream and react violently. Serum citrate levels of specimens taken a few moments later exceeded 8 mg./100 ml. Rabbit 6 reached such a level be-

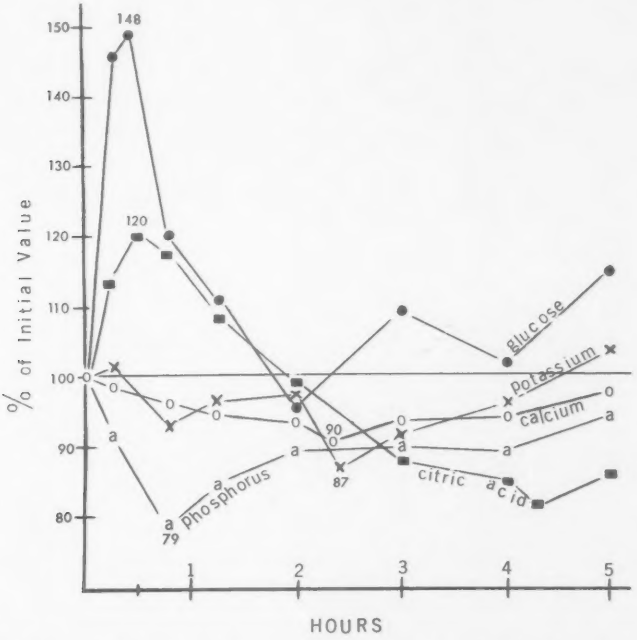


**Fig. 7.** Response of blood serum levels to 0.14 mg./kg. of crystalline glucagon in fasting male rabbits. Mean values for 7 rabbits.

fore the fasting level (Table 5). This rabbit and Rabbit 6 (Table 4), whose fasting level was 7.72 mg./100 ml., were difficult to handle before the test. Thus fright alone will raise serum citrate levels markedly in rabbits. We believe this is an adrenaline, coupled with an ACTH, effect.

In Fig. 5 and 8, in which the effects of hydrocortisone and parathormone are shown, the small elevations of citrate noted at the outset of the experiment may have been due merely to the pain and stress of the initial injection. Transient changes in citrate levels must be therefore interpreted cautiously. For example, in one experiment it was reported

**Table 9.** Effect of Intramuscular Parathormone* on Serum Ca, K, P, Citrate, and Glucose Levels in Rabbits

| *Rabbit No.* | *Component* | *Fasting* | *Time after collection of specimen (hr.)* | | | | | |
|---|---|---|---|---|---|---|---|---|
| | | | ½ | 1 | 2 | 3 | 4 | 5 |
| | Ca | 13.14 | 13.26 | 13.32 | 13.90 | 14.0 | 13.84 | 13.05 |
| | K | 2.55 | 3.24 | 2.85 | 2.62 | 2.68 | 2.70 | 2.77 |
| 1 | P | 4.04 | 4.52 | 4.44 | 4.44 | 4.44 | 4.52 | 4.35 |
| | Citric | 5.66 | 5.59 | 5.66 | 5.59 | 5.49 | 5.59 | 5.38 |
| | Glucose | 118.0 | 103.5 | 102.0 | 118.0 | 105.5 | 118.0 | 124.0 |
| | Ca | 13.22 | 13.85 | 13.10 | 13.64 | 14.15 | 13.83 | 14.24 |
| | K | 3.01 | 3.05 | 2.93 | 2.90 | 2.95 | 3.15 | 3.15 |
| 2 | P | 4.83 | 4.87 | 4.70 | 4.99 | 5.12 | 5.12 | 5.07 |
| | Citric | 5.34 | 5.37 | 5.54 | 5.57 | 5.50 | 5.64 | 5.67 |
| | Glucose | 82.7 | 84.9 | 81.1 | 91.9 | 91.9 | 96.2 | 115.7 |
| | Ca | 13.0 | 12.53 | 13.45 | 13.62 | 13.63 | 13.77 | 13.30 |
| | K | 3.46 | 3.41 | 3.58 | 3.58 | 3.27 | 3.28 | 3.57 |
| 3 | P | 4.0 | 3.35 | 3.14 | 3.14 | 3.23 | 3.27 | 3.57 |
| | Citric | 5.65 | 6.04 | 6.08 | 5.34 | 5.09 | 5.13 | 4.94 |
| | Glucose | 119.4 | 127.2 | 135.2 | 156.0 | 122.4 | 130.8 | 123.0 |
| | Ca | 13.42 | 12.70 | 13.45 | 13.32 | 13.45 | 13.94 | 13.38 |
| | K | 3.19 | 3.13 | 3.23 | 3.23 | 3.79 | 3.00 | 2.93 |
| 4 | P | 4.29 | 3.49 | 4.16 | 3.74 | 3.37 | 4.66 | 4.16 |
| | Citric | 4.73 | 5.67 | 6.50 | 5.64 | 5.15 | 4.73 | 4.88 |
| | Glucose | 142.2 | 119.2 | 119.2 | 116.4 | 96.5 | 134.6 | 125.0 |
| | Ca | 13.1 | 14.55 | 13.8 | 14.52 | 14.67 | 14.40 | 14.20 |
| | K | 4.02 | 4.74 | 4.22 | 4.23 | 4.16 | 4.17 | 4.44 |
| 5 | P | 3.96 | 3.60 | 4.40 | 3.92 | 4.28 | 4.60 | 4.32 |
| | Citric | 8.0 | 8.5 | 8.35 | 7.28 | 6.0 | 6.10 | 6.51 |
| | Glucose | 132.0 | 110.0 | 132.0 | 108.0 | 111.0 | 100.0 | 110.0 |
| | Ca | 12.30 | 12.61 | 12.32 | 12.36 | 12.0 | 12.39 | 12.18 |
| | K | 4.02 | 3.90 | 3.67 | 3.60 | 3.66 | 3.63 | 4.02 |
| 6 | P | 6.04 | 5.87 | 5.83 | 5.95 | 5.36 | 5.19 | 5.19 |
| | Citric | 3.83 | 4.12 | 3.98 | 3.98 | 3.54 | 3.44 | 3.03 |
| | Glucose | 115.0 | 139.5 | 139.0 | 153.5 | 155.5 | 144.5 | 157.0 |

*Dosage, 100 U./kg.

that a maximum citrate level was reached 4 hours after nephrectomy (*23*). The question may be asked, "Is this a stress phenomenon due to ACTH as in Fig. 1?"

The rise in citrate with ACTH has also been observed in humans by others (*18*). The fall in citrate with insulin has also been confirmed (*1*). The transient rise in serum citrate level has been observed before on parathormone administration (*37*). One experimentor stated that

with parathormone the increase in citrate precedes the rise in calcium (*20*). This can be seen in Fig. 8. This transient rise may also have been caused by stress and may be so interpreted. Calcium levels did not rise significantly during that time and could not be responsible for

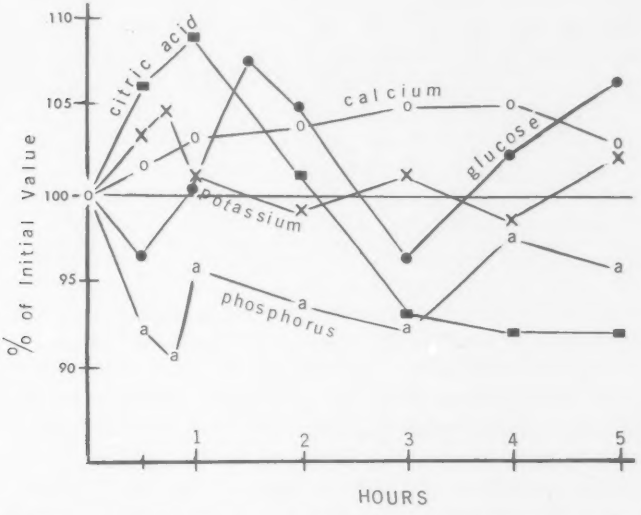


**Fig. 8.** Response of blood serum levels to 100 U./kg. of parathormone intramuscularly in rabbits. Mean values for 6 fasting male rabbits.

the elevation. In another experiment reported (*6*), calcium salts injected intramuscularly caused a rise in serum citrate levels, but so did saline solutions. Here again the stress of the injection may have been measured and not the effect of the material injected.

In view of the above, one may say that an immediate rise in citrate is caused by acute stress to the rabbit. This may be an adrenaline effect. Maintenance of a high citrate level over a longer period is probably an ACTH effect. Interpreting our early experiments (*51*) and those of others in which individuals who had been repeatedly placed under stress would not respond with a drop in citrate levels on glucose administration (*39*), we now believe that this indicated a hypertrophied stress mechanism. The elevation in citrate caused by adrenaline and ACTH overpowered the insulin effect in these individuals. It is interesting to note that even in some normal children when glucose was ad-

**Table 10.** Effect of Intramuscular *l*-Adrenaline* on Serum Ca, K, P, Citrate, and Glucose Levels in Normal Male Children

| *Child No.* | *Age, weight* | *Component* | *Fasting* | *Time after collection of specimen (hr.)* ½ | *1* | *2* |
|---|---|---|---|---|---|---|
| | | Ca | 9.13 | 9.48 | 9.11 | 9.08 |
| | 17 mo., | K | 4.0 | 3.51 | 3.84 | — |
| 1 | 14 kg. | P | 4.90 | 3.86 | 4.28 | 4.65 |
| | | Citric | 2.53 | 2.96 | 2.60 | 2.67 |
| | | Glucose | 78.8 | 129.6 | 99.36 | 76.61 |
| | | Ca | 9.82 | 9.51 | 9.58 | 9.80 |
| | 18 mo., | K | 4.38 | 3.19 | 3.60 | 3.19 |
| 2 | 16 kg. | P | 5.31 | 5.0 | 5.31 | 5.45 |
| | | Citric | 2.14 | 2.76 | 2.35 | 2.28 |
| | | Glucose | 80.5 | 176.0 | 190.5 | 120.5 |

*Dosage, 0.05 mg./kg.

ministered in these early experiments (*49*), a transient rise in citrate was noted before the fall. This could be explained in the same way, the stress on these children being greatest at the start of the experiment.

It must be emphasized at this point that changes in endogenous citrate levels under the influence of ACTH are of marked significance. The body is well equipped to metabolize citrate rapidly. For example, in an earlier study on the fate of citrate in erythroblastotic infants (*68*), it was demonstrated that citrate administered to the human disappeared rapidly. In these infants, weighing of the order of 2–3 kg., the citrate concentration of the blood infused was approximately 340 mg./100 ml. One thousand milliliters of blood was infused into these infants, and a like amount was removed. Less than 6% of the infused citrate could be accounted for in the infant at the end of the transfusion. Blood levels quickly returned to normal.

### Calcium

Of greatest interest to us was the effect of the hormones on ionized calcium and potassium levels. Since no direct practical method exists for measuring the ionic concentrations of these materials, we have to be content with assaying for total concentration.

It must be kept in mind that slight changes in total concentration can represent a significant change in ionic concentration. Since approximately 40% of the serum calcium is ionic, an absolute drop of 1 mg./100 ml. should be compared with 4 mg./100 ml. as an estimated initial concentration and not 10 mg./100 ml., the initial total concentration in the

human. This now represents a 25% drop. If this is coupled with a significant elevation of citrate or phosphate levels, it can readily be shown that a drop of as much as 50% in ionic concentration can be reached, if there is no change in blood *p*H.

Table 1 indicates that the drop in serum calcium with ACTH was rapid and consistent. In this effect, in the rabbit, we have confirmed our earlier work. Unit for unit, it made little difference whether we used a preparation containing 1 U. per milligram, as in our earlier work 11 years ago, 25 U. per milligram, as in the present study, or a commercial preparation from a different source; the effect was the same. Thus this is probably an effect of ACTH and not some other hormone impurity, unless the impurity was not separated from ACTH on the oxycellulose column. For the present, one must conclude that in the rabbit ACTH is a prime factor in the control of calcium homeostasis. The profound changes in calcium concentration coupled with a marked rise in citrate level, as observed in Fig. 1 and Table 1, can well account for the tendency of these rabbits to have convulsions. The mechanism here probably comprises the following sequence of events. Decreased ionic calcium results in decreased cardiac output and decreased blood supply to the brain. When stimulated, increased activity consumes the oxygen brought to the brain faster than it can be replaced by the decreased cardiac output. Anoxia ensues, followed by convulsions.

It is of interest that in a recent monograph ACTH is not considered at all in calcium homeostasis (*10*).

A drop in serum calcium levels coupled with a rise in citrate levels as observed in Fig. 1 would tend to increase neuromuscular activity (*43*). ACTH has been reported as a thalamacortical activator in rabbits when tested by electrical stimulation (*45*). This observation may reflect the drop in calcium ion concentration due to the ACTH administered.

The question immediately arises as to why this effect is not observed in the human (Table 2, Fig. 2). Examination of the individual cases in Table 2 indicates that the calcium levels fluctuate while they move upward, finally reaching a level higher than the fasting one. This marked difference in behavior between the human and the rabbit requires an explanation which cannot be given at present. There are certain obvious species differences between the rabbit and the human as far as their stress mechanism is concerned. The major corticosteroid in the rabbit and rat is corticosterone and not hydrocortisone as in the human

(*55*). In preliminary experiments with corticosterone (4 rabbits), only a slight decrease of the calcium level was observed even when massive doses (25 mg. per kilogram) were injected intramuscularly. Thus ACTH does not act through corticosterone in lowering calcium levels.

A lowering of serum calcium levels on ACTH administration was observed in children who had exhibited febrile convulsions and in 1 patient with hypospadias (Table 3, Cases 4, 6, 7, 8, and 2). The results in Case 7 were particularly puzzling. With the X-ray spectrometer, three samples from each serum are placed on filter paper and allowed to dry. The papers are then read in the spectrometer. The technic is nondestructive and the papers can be read again and again. The results of the analysis are accurate to $\pm$ 2% (2 $\sigma$). Yet, with a result as low as 2.96 mg./100 ml., the child was not observed to have convulsions. Since the child became very pale, was sweating, and seemed to be having difficulty breathing, the 5-hr. sample was not drawn. For obvious reasons, we cannot repeat this test at this dosage in this child. Fasting calcium levels in this child 24 hr. before and after the test were normal. The fact that some children act in an anomalous manner with respect to the effect of ACTH on serum calcium levels is a subject we are continuing to investigate.

An interesting observation is that histamine drops serum calcium levels for 1, 2, and sometimes 3 hr. in man (*38*). This needs to be investigated in relation to the ACTH and adrenaline effect described above. If histamine extracted from muscle was causing the drop in calcium levels, then all the material injected intramuscularly would have caused a drop in calcium levels due to muscle trauma. Since this was not true, the action of histamine may be secondary to its action on the stress mechanism. Other hormones are also probably involved in calcium homeostasis under some special conditions. Estrogens cause a marked rise in calcium levels in guinea pigs (*14*). Male steroid hormones do not have so marked an effect.

In the rabbit, acute control of calcium levels is controlled by a balance between at least two hormones. It is apparent from Table 4 and Fig. 3 that ACTH does not act through nor is antagonized by adrenaline. Instead, adrenaline acts on calcium levels like a small dose of ACTH. Most likely, this action is due to release of endogenous ACTH by adrenaline. Neither does the ACTH act, in this respect, by release of corticosterone or hydrocortisone. Corticosterone and hydrocortisone are not antagonists to ACTH with respect to serum calcium. One

naturally suspects that the antagonist would be a hormone from the parathyroids.

There is ample evidence for an interrelationship between the pituitary and the parathyroids. Parenchymal parathyroid cells decrease in size after hypophysectomy (*69*). There is a 42% atrophy of the parathyroids only 8 days after hypophysectomy (*13*). The parathyroid gland responds to lowered serum calcium levels with increased secretion of hormone (*50*). Thus the action of the pituitary on the parathyroids may be directed by some mechanism in control of serum calcium levels.

In the dog, it has been observed that on removal of the pituitary there is a transitory decrease of calcium levels. This may be due to discharge of ACTH during surgery. Subsequently, levels are elevated, and intravenously administered calcium circulates significantly longer in the serum than in normal dogs (*25*). This observation may suggest that the action of ACTH in lowering calcium levels may be on cell permeability as suggested for the action of insulin on glucose. Citrate may be involved, in that a rise in citrate would complex calcium and permit the less polar complex to move into the cells. This is supported by the fact that citrate causes the calcium to become readily ultrafilterable (*61*). Calcium citrate moves through the intestinal wall much more rapidly than calcium phosphate (*59*). Thus it is suggested that in our present studies the citrate levels observed, as in Fig. 1, are an underestimate of the amount poured into the serum, a good deal of it having moved into the cells with the calcium.

This concept may explain some observations made in earlier experiments. For example, if an extract of the anterior pituitary is injected into guinea pigs for 9 days, a significantly higher serum calcium is found in these animals at the end of the experimental period than in controls (*24*). We would explain this by saying that a constant lowering of calcium levels by ACTH stimulates the parathyroids to produce more parathormone. When injections are stopped, the induced hyperparathyroidism becomes evident.

Our present experiments with parathormone (Fig. 8) are inconclusive. Elevation of calcium is too slow to act as a rapid antagonist for the prompt action of ACTH in lowering calcium levels, even though we gave large doses to these animals (100 U. per kilogram). The material may have been poorly absorbed. Intravenous experiments need to be done simultaneously with ACTH and parathormone administration to check this point. Intravenous experiments by others suggest

parathyroid action on calcium is rapid enough to act as an antagonist to ACTH (*12*).

Recently (*11*) a report appeared demonstrating a transient drop of serum calcium (within 20 min.) in intact dogs when commercial parathormone (Lilly) was injected intravenously, 300–1000 U. in less than a minute. Subsequently, serum calcium levels rose above fasting levels. We had noted similar effects in 2 of 6 rabbits (Table 9, Rabbits 3 and 4), using the same material intramuscularly. We attributed this drop to adrenaline and ACTH released by the stimulus of the initial injection. This might explain the results reported since these experimenters suddenly injected 300–1000 U. of a foreign protein, parathormone. While a hypocalcemia factor may be secreted by the parathyroids, this experiment has not eliminated the possibility that this is an effect of the pituitary, provided dogs behave like rabbits with ACTH and adrenaline. It would be interesting to see if this effect would be observed in dogs with the pituitary and adrenal removed.

### Potassium

A major force in the homeostatic control of serum potassium levels is adrenaline. Potassium drops to the same degree and at about the same time with insulin, adrenaline, and ACTH (Fig. 1, 3, and 6). Lowering of serum potassium with adrenaline has been noted in rats (*16*), humans (*63*), and rabbits (*44*). This drop is not due to passage into the urine because potassium urinary output actually decreases with adrenaline (*31, 35*).

In intact, adrenalectomized, adrenal-enucleated, and hypophysectomized rats, adrenaline lowers potassium levels. Insulin reduces potassium levels only in intact rats (*17*). This study suggests that the effect of ACTH (Fig. 1) and insulin (Fig. 6) on potassium levels is probably secondary to the stimulus of endogenous adrenaline secretion. A drop in potassium levels with ACTH has been observed before by others (*2, 19, 58*).

Noradrenaline raises potassium levels in rabbits (Fig. 4). This has also been observed in dogs (*46*). One may speculate that the action of noradrenaline is as an adrenaline antagonist in this respect.

An interesting observation (*15*) is that adrenaline reduces potassium levels more in epileptics than in normal humans. This suggests that an antagonist to the action of adrenaline is absent in the epileptic. Our experiments do not indicate what this antagonist may be. The effect of noradrenaline seems to be too small for it to be the factor.

Hydrocortisone and parathormone show little effect on potassium levels in our experiments. A rise in serum potassium in man after intravenous infusion of hydrocortisone has been reported (*34*) in spite of increased urinary excretion of the element. Glucagon behaves in cats essentially as it does in rabbits (Fig. 7). A transient mild hyperkalemia is followed by hypokalemia (*70*).

### Phosphate

The control of serum phosphate levels is affected by several factors acting differently. Adrenaline, noradrenaline, insulin, glucagon, ACTH, and parathormone (Fig. 1, 3, 4, and 6–8) all cause serum phosphate levels to drop significantly. Thus whether glucose is moving into the cells or out of the cells, the phosphate levels drop. In an isolated rat diaphragm, it has been demonstrated that insulin causes phosphate to move into the cells (*56*).

Since adrenaline constricts the capillaries and decreases urinary output, the phosphate must also be moving into the cells with this substance. With adrenaline the minimum is reached an hour later than with insulin. It is therefore suggested that the adrenaline effect is indirect and acts by inducing insulin secretion. Drop in phosphate on adrenaline administration has also been observed in man (*71*). This effect is also noted in the case of the 2 children (Table 10) although the drop is slight in the second child.

In toads, hypophysis removal results in a drop in serum phosphate (*26*). This is not only an effect of ACTH as in Fig. 1 but also an effect produced by lack of the growth hormone, somatotropin. In our experiments hydrocortisone had no acute significant effect on serum phosphate levels. In humans, hydrocortisone is reported to drop phosphate levels (*40*). The effect of parathormone on the kidneys in stimulating excretion of phosphate is well documented (*54*). The effect of this hormone on the serum levels in the rabbit can be seen in Fig. 8.

There appear to be two modes of removal of phosphorus from the serum, namely the movement of phosphate into the cells with insulin and through the kidneys with parathormone. A means of maintaining elevated serum phosphate levels is not demonstrated in our experiments. It should be noted that with ACTH (Fig. 2) in the human, the phosphate levels as well as the calcium levels move in the opposite direction to that observed in the rabbit.

Our experiments do not indicate a mechanism for maintaining elevated serum phosphate levels. Leaching of phosphate from the bone

and tissues by an undersaturated serum is probably one factor. Absorption from the intestine on an adequate intake of calcium and phosphorus and the role of vitamin D (still not clear) are exogenous factors. In the human, lack of vitamin D results in reduced serum phosphate levels. In the rat the serum phosphate levels are increased in this condition. This is explained by reduced renal absorption of phosphate by the human kidney but not in the rat kidney (*10*). A rapid positive homeostatic mechanism probably exists that moves phosphate quickly out of the cell in time of need and limits kidney excretion. While parathormone moves phosphate out of the cells, its effect on the kidney is such that a net lowering of serum phosphate results. This system still needs to be clearly defined.

In the light of these experiments and the literature documented, one may draw the following limited conclusions: In the rabbit, ACTH and parathormone are prime movers of calcium into and out of the serum. Potassium movement into the cells is acutely affected by adrenaline. Insulin effect is probably secondary to its stimulation of adrenaline secretion. Phosphate movement into the cells with reduction of serum phosphate is induced by insulin. Parathormone also reduces serum phosphate, by acting on the kidneys. Citrate elevation is produced by ACTH, adrenaline, and glucagon. Insulin is a balancing factor, lowering serum citrate levels. Stress causes a rise in citrate levels, probably due to the adrenaline and ACTH effects. Serum glucose levels are controlled by a balance between insulin, which drives glucose into the cell, and glucagon and adrenaline, which work in the opposite direction. Cortisone is not an insulin antagonist but has a long-range effect. The ACTH effect is similar to that of cortisone, probably by adrenal cortical stimulation.

Only in the case of serum citrate is the response of the human similar to the rabbit when ACTH is administered intramuscularly. With this hormone calcium, potassium, and phosphorus levels tend to rise instead of to fall as in the rabbit. The curve for glucose is the inverse of that seen in the rabbit. In the rabbit a fall in glucose is followed by a rise to hyperglycemic levels. In the child a rise is followed by a fall.

It is suggested that calcium, potassium, and phosphate levels are in critical homeostatic control by the various hormones of the living organism.

Mean serum levels in fasting rabbits for the constituents studied were found to be as follows: calcium, 12.75 mg./100 ml. ± 1.90; potassium, 3.66 mEq./L. ± 1.03; phosphorus, 4.55 mg./100 ml. ± 1.80; citric

acid, 4.88 mg./100 ml. ± 1.82; and glucose, 116.0 mg./100 ml. ± 54.0. These results are the mean values found in 55 rabbits, and the ranges indicated are ± 2 S.D.

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# Variation of Protein Content of Urine in a 24-hour Period

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**Variation of the protein content in urine of patients known to excrete protein in excessive amounts was studied. Variation in different specimens obtained from the same patient on a particular day and from day to day were studied. A total of 293 urine specimens excreted over a 3- to 6-day period by 25 patients were examined. No particular pattern of protein excretion was observed. The morning specimen was not the specimen with the highest protein content. This could occur at any time during the day. It is suggested that only a 24-hour collection can give a true indication of protein spillage.**

**A commercial paper strip test was compared with the qualitative sulfosalicylic acid reaction and a quantitative biuret procedure. The strip test is satisfactory for screening purposes except for highly colored urines, in which case the sulfosalicylic acid procedure is recommended.**

THE ANALYST of the clinical laboratories is often faced with the problem of variability in the protein content of casual specimens of urine from an individual patient. To the physician the question arises as to whether these variations are real or due to laboratory error. The purpose of the study reported below was to explore the extent of variation in protein output in different specimens voided by patients with various intensities of kidney disease resulting in proteinuria.

Urine specimens were collected separately within a 24-hour period at the particular times that the patient chose to void. These were promptly analyzed for their protein content. The protein excreted for the 24-hour period was then obtained by totaling the amounts of

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protein in milligrams (mg./ml. × volume) obtained for each specimen.

The percentage of total protein excreted each time that the patient voided was then calculated from the total excretion for the 24-hour period.

This procedure was repeated for 3 or 4 days on the same patient, to measure at the same time variation in protein concentration in the same individual from day to day. Ten representative examples of values obtained are listed in Table 1.

In order to determine whether there was any particular period when a casual specimen might be taken with an optimum opportunity of detecting an abnormally high protein level, the data were distributed as shown in Table 2, the day being divided into 12 equal intervals and the number of patients voiding in each interval listed. The resulting data were further distributed by classifying in accordance with the range (percentage) of total daily protein excretion in that interval. The chi-square test was then applied to see whether the distribution for each protein level differed from any other. Simply stated, we sought to find if there was any significant difference in distribution between any one protein range and the distributions of frequency for the total number of specimens obtained. These data also are listed in Table 2.

Protein content of urine was determined quantitatively by precipitating the protein with tungstic acid and applying the biuret reaction to the precipitate. The values so obtained were compared with results obtained with two qualitative procedures generally used in clinical laboratories: the estimation of turbidity produced with sulfosalicylic acid, and the color obtained with Combistix test paper (Ames). These comparisons are tabulated in Tables 3 and 4.

## Procedure

### Collection of Specimens

Urine specimens were collected around the clock from patients known to excrete excessive amounts of protein, allowing the patient to void ad libitum. The volume of each specimen was measured and its protein content assayed by the three methods described below.

### Protein Determination (Qualitative)

The specimens were centrifuged. For the sulfosalicylic acid procedure (*1*) an equal volume of sulfosalicylic acid (3%) was mixed

with an equal volume of urine and allowed to stand for 3 min. A clear solution was considered negative; a faint cloud, a trace; and a heavier cloudy solution was rated 1-plus. A cloudy solution which had begun to settle was taken as 2-plus; a cloudy solution with a moderate amount of precipitate, 3-plus; and cloudy solution with a heavy precipitate, 4-plus.

For the Combistix reagent strip test (*2*) the paper was dipped in the urine and the color compared with the color chart supplied with the reagent.

### Protein Determination (Quantitative)

The biuret procedure (*3*) was used for this analysis, with Labtrol, a commercially available protein control, used as a standard. If the qualitative test showed 1-plus or less, 5 ml. of urine was added to a conical 15-ml. centrifuge tube. For 2-plus, 2 ml. of urine was added to a conical tube plus 3 ml. water; for 3-plus and 4-plus, 1 ml. urine plus 4 ml. water, and 0.5 ml. urine plus 4.5 ml. water, respectively. To each tube 5 ml. of tungstic acid was added. The tubes were mixed by inversion and centrifuged for 10 min. at 2000 rpm. The supernatant was decanted and the last drop touched off with gauze. To each tube 1.5 ml. water and 1.5 ml. biuret reagent was added. The solution was mixed by tapping and read on the Klett-Summerson Colorimeter with the 54 filter after 20 min. The blank comprised 1.5 ml. water and 1.5 ml. biuret reagent. The standard was prepared by washing 0.02 ml. protein standard (e.g., Labtrol) into 1.5 ml. water and adding 1.5 ml. biuret reagent.

### Calculations

The results were calculated as follows:

$$\frac{\text{Absorbance of unknown}}{\text{Absorbance of standard}} \times \frac{\text{mg./100 ml. protein in standard}}{250} \times \frac{\text{ml. urine used}}{5} = \text{mg./100 ml. protein in urine.}$$

### Reagents

*Sulfosalicylic acid (3%)* Three gm. of sulfosalicylic acid made up to 100 ml. with water

*Biuret reagent* One part of 2% copper sulfate mixed with five parts of 25% sodium hydroxide (w/v)

*Tungstic acid* Equal amounts of 0.15N sulfuric acid and 2.2% sodium tungstate mixed on the day of the test

*Combistix reagent strips* (Ames Co., Inc., Elkhart, Ind.)

*Labtrol* (Dade Reagents, Inc., Miami, Fla.)

## Discussion

In Table 1 the protein spillage in milligrams per 100 ml. is given for 10 patients during their waking hours.

From the table it is apparent that wide variation in protein content occurs from specimen to specimen. This variation has no predictable sequence. For example, in Patient 1, on the first day, the lowest level is obtained on the fifth specimen or near the end of the waking day. In the same patient on the fourth day, the highest level is reached at the end of the day. Individual values can range from negative to 4-plus on the same patient on the same day. This can be seen in Patients 5, 7, and 8 and, most dramatically, in Patient 10, where, on the first and second days values ranged from 1.1 to as high as 741 mg./100 ml.

It is commonly thought that the morning specimen contains the highest concentration of protein for the day. Out of a total of 36 days listed in Table 1, this occurs only 7 times. Thus, any specimen during the day may contain the highest concentration of protein.

The conclusion must be drawn that, if casual specimens are taken, then only repeated tests of each specimen voided can give reasonable indication of protein spillage. For quantitative assay of the total protein excreted, a 24-hour specimen needs to be collected. This was true for all the patients studied. The 10 cases listed in Table 1 are representative of the group observed.

From Table 2 it can be seen that there is no specific pattern which would permit selection of a specimen that would provide the greatest likelihood of finding the maximum protein concentration in the urine excreted. Thus, when the chi square test was applied, comparing distributions of each column with the column listing the total number of specimens obtained, no significant difference was observed.

The data of Table 3, where the protein concentration is compared to the turbidity observed with sulfosalicylic acid, readily show that this procedure is satisfactory for screening purposes. However, the technician will have some difficulty in distinguishing between a 1-plus and 2-plus value.

**Table 1.** PROTEIN LEVELS (MG./100 ML.) IN URINE SPECIMENS FROM THE SAME PATIENT

| | | *Specimen** | | | | | |
|---|---|---|---|---|---|---|---|
| *Patient* | *Day* | *1* | *2* | *3* | *4* | *5* | *6* |
| 1 | 1 | 223.2 | 221.1 | 122.9 | 121.8 | 90.9 | |
| | 2 | 158.1 | 312.6 | 271.3 | 189.0 | 235.8 | 140.0 |
| | 3 | 267.1 | 313.1 | 331.1 | 184.1 | 149.1 | |
| | 4 | 52.4 | 49.6 | 26.6 | 25.1 | 44.3 | 178.9 |
| 2 | 1 | 8.2 | 5.8 | 26.2 | 5.1 | 10.5 | 5.4 |
| | 2 | 5.8 | 1.4 | 2.2 | 4.0 | 5.2 | 3.8 |
| | 3 | 1.2 | 7.1 | 4.1 | 2.1 | 2.6 | 2.6 |
| | 4 | 6.2 | 8.5 | 8.8 | | | |
| 3 | 1 | 254.5 | 218.1 | 134.4 | 108.9 | | |
| | 2 | 108.9 | 116.2 | 130.7 | 134.4 | 78.1 | 58.1 |
| | 3 | 85.4 | 136.4 | 163.5 | 247.6 | 196.3 | 127.3 |
| 4 | 1 | 2.1 | 2.4 | 2.9 | | | |
| | 2 | 3.5 | 2.5 | 4.6 | 3.4 | 2.5 | 8.8 |
| | 3 | 3.5 | 35.4 | 39.7 | 7.4 | 6.0 | 23.4 |
| | 4 | 46.1 | 37.2 | 1.1 | | | |
| 5 | 1 | 3.6 | 1.6 | 2.0 | | | |
| | 2 | 83.2 | 2.6 | 1.3 | 1.6 | 4.0 | |
| | 3 | 3.6 | 4.3 | 5.0 | 3.5 | | |
| | 4 | 2.3 | 4.6 | | | | |
| 6 | 1 | 3.7 | 2.2 | 1.8 | 1.5 | 2.2 | 5.5 |
| | 2 | 2.2 | 1.8 | 5.9 | 4.6 | | |
| | 3 | 2.3 | 1.8 | 3.9 | 2.1 | 4.3 | 5.4 |
| | 4 | 7.2 | 9.0 | 5.9 | 3.9 | 3.9 | 6.1 |
| | 5 | 2.1 | 5.6 | 2.5 | 5.0 | 3.8 | 3.0 |
| 7 | 1 | 1.8 | 1.1 | 1.1 | 1.8 | 1.8 | |
| | 2 | 0.7 | 0.5 | 5.3 | 0.7 | 3.8 | |
| | 3 | 1.0 | 1.1 | 2.3 | 1.7 | 2.9 | |
| 8 | 1 | 1.1 | 0.5 | 1.8 | 2.5 | 5.1 | |
| | 2 | 2.1 | 1.1 | 1.4 | 2.3 | 0.7 | |
| | 3 | 1.9 | 3.8 | 4.2 | 6.9 | 10.7 | |
| 9 | 1 | 32.0 | 32.8 | 37.6 | 47.9 | 75.8 | |
| | 2 | 16.2 | 15.1 | 21.0 | 19.5 | 18.0 | |
| | 3 | 29.1 | 19.5 | 16.6 | | | |
| 10 | 1 | 2.8 | 1.1 | 741.7 | | | |
| | 2 | 425.3 | 2.8 | 5.8 | 5.4 | | |
| | 3 | 374.3 | 599.8 | 436.2 | 527.2 | | |

*Specimen 1 is the morning specimen; Specimen 6, the one obtained immediately before patient retired.

The use of the protein test paper (Table 4) is somewhat better in this respect, differences being more readily observed. However, in highly colored urine the test paper loses its value. For screening purposes this would be a preferred procedure, with the sulfosalicylic acid method used when the urine is highly colored.

**Table 2.** Distribution of Protein Spillage in Urine in Terms of Percentage of Daily Total Protein Output

| Time interval | Total specimens* | % of total urine specimens | No. of specimens obtained according to percentage of total output | | | | |
|---|---|---|---|---|---|---|---|
| | | | 0-9.9% | 10-19.9% | 20-29.9% | 30-39.9% | 40-49.9% |
| 12-1:59 A.M. | 11 | 3.7 | 2 | 2 | 3 | 3 | 1 |
| 2-3:59 | 13 | 4.4 | 3 | 5 | 2 | 1 | 2 |
| 4-5:59 | 23 | 7.8 | 4 | 12 | 4 | 2 | 1 |
| 6-7:59 | 23 | 7.8 | 6 | 8 | 6 | 3 | 0 |
| 8-9:59 | 17 | 5.8 | 5 | 7 | 2 | 3 | 0 |
| 10-11:59 | 42 | 14.3 | 18 | 7 | 8 | 7 | 2 |
| 12-1:59 P.M. | 26 | 8.8 | 13 | 5 | 4 | 2 | 2 |
| 2-3:59 | 35 | 11.9 | 15 | 3 | 8 | 6 | 3 |
| 4-5:59 | 26 | 8.8 | 5 | 13 | 5 | 2 | 1 |
| 6-7:59 | 37 | 12.6 | 10 | 15 | 7 | 3 | 2 |
| 8-9:59 | 28 | 9.5 | 12 | 7 | 6 | 3 | 0 |
| 10-11:59 | 12 | 4.0 | 3 | 3 | 3 | 2 | 1 |
| Total | 293 | 99.4 | 96 | 87 | 58 | 37 | 15 |

*Where patient voided more than once in 2-hour period, specimens were pooled and entered as one.

It is of interest to compare the values given by the manufacturer with those obtained in the quantitative procedure. On the test paper color chart, 1-plus, 2-plus, 3-plus and 4-plus are rated as 30, 100, 300, and over 1000 mg./100 ml., respectively. We observed 32.6, 95, and 234 as the mean for the first three levels. In our study we did not encounter enough cases of the order of 1000 mg./100 ml. to make a statistical evaluation at this level.

Our general conclusions from this study are as follows.

1. Protein concentration in urine will vary widely from specimen

**Table 3.** Comparison of Qualitative Sulfosalicylic Acid Turbidity Procedure with Quantitative Biuret Method

| Number of samples in group | Reading | Mean conc. by biuret (mg./100 ml.) | Range (2 × S.D.) | t value* | Excess of t value for significance at 1% level |
|---|---|---|---|---|---|
| 18 | 0 | 4.3 | ± 1.96 | | |
| 31 | Trace | 6.6 | ± 2.32 | 6.9 | 2.7 |
| 21 | 1+ | 60.4 | ±25.72 | 22.7 | 2.7 |
| 17 | 2+ | 83.5 | ±39.28 | 4.2 | 2.8 |
| 18 | 3+ | 172.2 | ±47.14 | 11.6 | 2.8 |

*Comparing means of adjoining groups; based on combined standard deviation for groups indicated.

Table 4. Comparison of Qualitative Combistix Reagent Strips Method with Quantitative Biuret Procedure

| *Number of samples in group* | *Reading* | *Mean conc. by biuret (mg./100 ml.)* | *Range (2 × S.D.)* | *t value** | *Excess of t value for significance at 1% level* |
|---|---|---|---|---|---|
| 173 | 0 | 0.6 | 0.08 | | |
| 48 | Trace | 7.6 | 2.16 | 85.4 | 2.6 |
| 17 | 1+ | 32.6 | 15.34 | 21.7 | 2.7 |
| 15 | 2+ | 95.0 | 47.39 | 10.0 | 2.8 |
| 64 | 3+ | 234.0 | 58.04 | 16.6 | 2.7 |

*Comparing means of adjoining groups; based on the combined standard deviation for groups indicated.

to specimen during a 24-hour period and from day to day in a patient excreting excessive amounts of protein.

2. No fixed pattern of protein excreted is observed and there is no best time to take a casual urine specimen for screening purpose. Total excretion can be evaluated only from a 24-hour specimen and total excretion will vary widely from day to day.

3. The strip test paper (Albustix or Combistix) is satisfactory for estimating the protein content of casual specimens for screening purposes, except in highly colored urines. In these cases the sulfosalicylic acid turbidity procedure is satisfactory, but it is less so in estimating semi-quantitatively the protein concentration. For quantitative assay of 24-hour urine specimens, the biuret procedure is convenient and accurate.

No attempt has been made in this paper to discuss the various etiologies of proteinuria, which is, of course, a subject in itself. We were here interested only in demonstrating the great variation in protein spillage that occurs in patients who may have anything from primary renal disease to congestive heart failure as a cause of this proteinuria. We are, of course, aware of the many physiologic causes of change in amount of urinary protein such as exercise, emotion, cold bathing, and postural changes, but purposely did not exclude any patient who might have had any of these factors operating, since these might very well precede the taking of a casual specimen in any patient.

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# Stationary Phase as Color Reagent in Glass Paper Chromatography of Estrogens

*Emanuel Epstein and Bennie Zak*

**A new technic has been described for the chromatographic separation of estrogens. It includes the use of a dual-purpose system where the impregnating material of the stationary phase (p-toluene sulfonic acid) is used as a color reagent after the separation is complete. This novel approach precludes the need for a spraying or dipping step to achieve the desired color reaction. Quantitation by densitometry and fluorescence is also detailed.**

CELLULOSE PAPER CHROMATOGRAPHY of the estrogens has been investigated extensively and several technics employ this medium (*1–5*). However, some disadvantageous factors involved in these determinations include lengthy chromatographic development and the insensitivity, nonspecificity, or destructive quality of detection reagents employed (*6–8*). Also, the analyst has been forced either to elute the estrogens after chromatography for applying the Kober reaction, or to resort to laborious column chromatography for a technic which can yield adequate results.

A recent investigation of steroids by glass paper chromatography demonstrated speed of development and separation (*9*). Sulfuric acid spray was used to detect the steroids by color, and further heating charred the steroid spots for a permanent record of the separation. Others continued this investigation with the aim of semiquantitating the charred steroid spots by comparison analysis (*10*).

In the present work a new simplified approach with a reasonable measure of specificity for the separation and quantitation of estrogens on glass paper is reported. A p-toluenesulfonic acid (PTSA) spot test technic (*11*) was applied to increase the facility of operation and to

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achieve selectivity and sensitivity of the completed chromatogram. Glass paper strips were impregnated with PTSA. This compound then served dually as stationary salt phase during chromatography, and as the color development reagent on moderate heating of the chromatogram. Heating estrogens in the presence of PTSA resulted in a controlled reaction in which red colors and yellow to red fluorescences were exhibited (*11*). Unlike charring with sulfuric acid, the strip has spectral characteristics which allow handling and instrumental analysis of the steroid colors and fluorescences. The following work illustrates and applies this technic.

## Procedure

### Reagents

1. *Impregnation solution* Prepare fresh 4% solution of PTSA in acetone.
2. *Developing reagent* Spectral grade benzene was saturated with PTSA by adding 1–2 gm. to 100 ml. of benzene and allowing it to become saturated. This eluent can be kept for several days without deterioration.
3. *Equilibration solution* Prepare a 12% solution of reagent grade methanol in spectral grade benzene.
4. *Pure estrogens* Estradiol-17 beta and estriol solutions were made from USP steroid reference standards, No. 5003 and 5007, respectively, while estrone standard solutions were made from pure, crystalline estrone. The standard solutions were made up in methanol so that 10 $\mu$L. contained the desired amount of steroid.

### Separation of Estrogens

The glass paper strips (16 × 1.5 in.)* were impregnated with PTSA by slowly drawing them through the impregnation solution with the aid of forceps, utilizing a "no touch technic" (*12*). Excess fluid was expressed from the strips by touching the end of the strip to the edge of the dipping tray. The strips were suspended with stainless steel clips and allowed to dry. If the strips were not used within an hour, they were stored in a covered stainless steel tray over indicating silica gel, a step especially advisable during humid days. The strips are good for several days before use if kept dry. A light pencil mark was made 7 cm. from the bottom of the freely suspended strip to mark the side

*No. 934 AH; H. Reeve Angel & Co., Clifton, N. J.

of sample loading. Ten microliters of the methanolic steroid was applied to that site with a Lang-Levy pipet, with the no-touch technic carefully adhered to both at this point and at all further steps of the procedure. (Dust and other impurities must be avoided in carrying out this technic.)

The loaded strips were inverted, transferred to the chromatography tank with the aid of forceps, and anchored in the dry solvent trough by means of heavy Teflon rods cut to 1–1.5-in. lengths. The chromatography tank (6 × 18 in.) was equilibrated for one hour with about 25 ml. of the equilibration solution contained in a petri dish in the bottom of the tank. The closed tank containing the glass paper strips was allowed to equilibrate for an additional 30 min. Developing reagent was then introduced into the solvent trough and the descending chromatographic process allowed to proceed to within 8 cm. of the lower end. This procedure took about 40 min. At the end of development, the strips were removed with forceps and suspended by the lower, unwet end until the benzene evaporated. The dry strips were mounted on a metal drying rack* placed in a 120° oven and heated for 10 min. The strips were transferred to a stainless steel tray containing dry indicating silica gel until they were analyzed. In this work the three estrogens were loaded on one strip in equal and varying amounts to obtain $R_f$ values and to construct calibration lines. The fact that the three estrogens travel in an independent way is indicated in Table 1, where estrogens were chromatographed separately and in combination.

## Instrumentation

### Fluorometry

A Turner fluorometer, Model 111,† with the chromatography attachment was used in this study. Primary light for 546 mμ was isolated with a No. 58 and a No. 1-60 filter combination; secondary light was limited to radiation above 579 mμ with a No. 23-A filter. Readings were made at the peak transmissions using a sensitivity (amount of incident light) of either 1× or 3×. $R_f$ values were calculated using the circular scale affixed to the drum mechanism. The solvent front was located by the small amount of fast-running fluorescent material that is normally present in the glass paper.

*Spinco Division, Beckman Instruments, Inc., Palo Alto, Calif.
†American Instrument Co., Washington, D. C.

Table 1. $R_f$ Values of Three Estrogens Run Singly and in Mixtures

| *Run* | *Estrone* | *Estradiol-17 β* | *Estriol* |
|---|---|---|---|
| 1 | 0.77 | — | — |
| 2 | 0.78 | 0.28 | 0.02 |
| 3 | — | — | 0.02 |
| 4 | — | 0.27 | — |
| 5 | — | 0.26 | — |
| 6 | 0.76 | — | — |
| 7 | 0.77 | 0.25 | 0.02 |
| 8 | 0.77 | 0.25 | 0.02 |

### Densitometry

An Analytrol* recording and integrating densitometer was used retaining the original B-5 cam and 500 mμ filters provided with the instrument. After the estrone spot had been scanned the instrument was stopped and the strip moved manually to a point just before the estradiol-17 beta spot and the readings continued. This enabled all three estrogens to be recorded on one chart. These spots were uniformly smaller than 11 mm. in diameter. Therefore, to scan such a small portion of the strip, the 4-mm. slit was masked with black electrician's tape to allow an exposure of only 11 mm. of the glass paper strip. If this was not done, the sensitivity was greatly decreased.

## Discussion and Results

The method presented here has several important advantages over previously reported glass paper chromatographic methods (*9, 12*);

1. There is no necessity for applying an additional detection agent since the impregnated stationary phase also acts as the color-developing agent.
2. The finished chromatogram has properties which allow handling and quantitation by instrumental means.
3. The possibility of measuring colors and fluorescences adds desirable specificity and increased sensitivity to this type of analysis.

### Developing System

The use of methanol with benzene to equilibrate the atmosphere of the chromatography chamber had a surprising effect on the separation characteristics of the estrogens, as shown in Fig. 1. Without methanol in the benzene, estrone and estradiol-17 beta were separated

*Spinco Division, Beckman Instrument Co., Palo Alto, Calif.

but occurred in the vicinity of the solvent front. With increasing amounts of methanol in the benzene, estrone, and estradiol-17 beta exhibited a greater degree of separation while estriol was relatively unaffected by this modification. A concentration of 12% methanol in benzene was arbitrarily selected for the separation system.

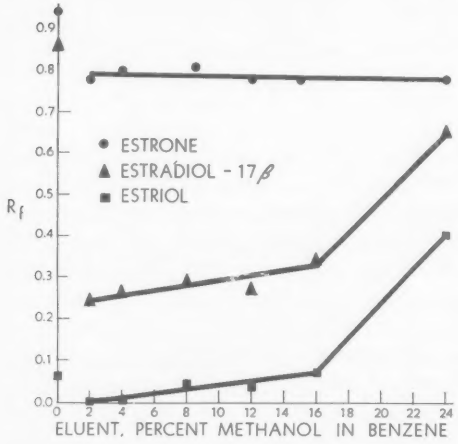


**Fig. 1.** Effect on $R_f$ of varying the methanol content of benzene equilibrating solution.

### $R_f$ Values

Table 2 illustrates the values obtained with six runs of 5–6 samples each of estrone and estradiol-17 beta. Within the same run in the 1 to 5-$\mu$g. range the spread of $R_f$ values was within 0.03, while the spread between separate runs was within 0.04. The estrogens were present as mixtures in both equal and unequal amounts. Distances were measured from the center of each spot, while the solvent front was located by the occurrence of fluorescent impurities in the paper, which traveled with the front. Table 2 also shows a somewhat greater spread of $R_f$ in the 0.25- to 1.0-$\mu$g. range. But, despite several aberrant results which gave rise to this greater spread, the average $R_f$ values compared well with the 1- to 5-$\mu$g. range values.

Figure 2, a developed estrogen chromatogram, illustrates the favorable $R_f$'s in well-differentiated separations, with an absence of tailing in the spots. The chromatographic development time was 40 min., which allowed the solvent front to traverse a distance of 27 cm. from the starting line.

**Table 2.** $R_f$ VALUES* FOR ESTRONE AND ESTRADIOL-17-BETA FROM MIXTURES

| | 0.25- to 1.0-μg. Range | | | | 1- to 5-μg. Range | | |
|---|---|---|---|---|---|---|---|
| Run† | Average $R_f$‡ | $R_f$ Spread | S.D. | Run | Average $R_f$ | $R_f$ Spread | S.D. |
| 1 E | 0.76 (5) | 0.75-0.77 | 0.007 | 1 E | 0.74 (5) | 0.74-0.76 | 0.008 |
| D | 0.25 (5) | 0.24-0.27 | 0.013 | D | 0.25 (5) | 0.24-0.27 | 0.014 |
| 2 E | 0.75 (6) | 0.73-0.76 | 0.012 | 2 E | 0.76 (6) | 0.76-0.78 | 0.008 |
| D | 0.25 (6) | 0.23-0.28 | 0.019 | D | 0.27 (6) | 0.26-0.28 | 0.009 |
| 3 E | 0.73 (6) | 0.71-0.76 | 0.022 | 3 E | 0.76 (5) | 0.75-0.78 | 0.013 |
| D | 0.21 (6) | 0.18-0.23 | 0.021 | D | 0.26 (5) | 0.26-0.27 | 0.004 |
| 4 E | 0.76 (5) | 0.76-0.77 | 0.004 | 4 E | 0.76 (5) | 0.74-0.77 | 0.013 |
| D | 0.26 (5) | 0.25-0.28 | 0.013 | D | 0.25 (5) | 0.25-0.27 | 0.009 |
| 5 E | 0.74 (5) | 0.73-0.76 | 0.0011 | 5 E | 0.76 (5) | 0.75-0.78 | 0.012 |
| D | 0.26 (5) | 0.23-0.33 | 0.040 | D | 0.25 (5) | 0.24-0.27 | 0.011′ |
| 6 E | 0.77 (4) | 0.77-0.79 | 0.010 | 6 E | 0.76 (5) | 0.76 | 0 |
| D | 0.26 (4) | 0.25-0.27 | 0.011 | D | 0.26 (5) | 0.26 | 0 |

*$R_f$ values for estriol are not shown as they are uniformly at the starting point.
†E indicates estrone; D, estradiol-17 beta.
‡Parentheses represent number of chromatograms averaged.

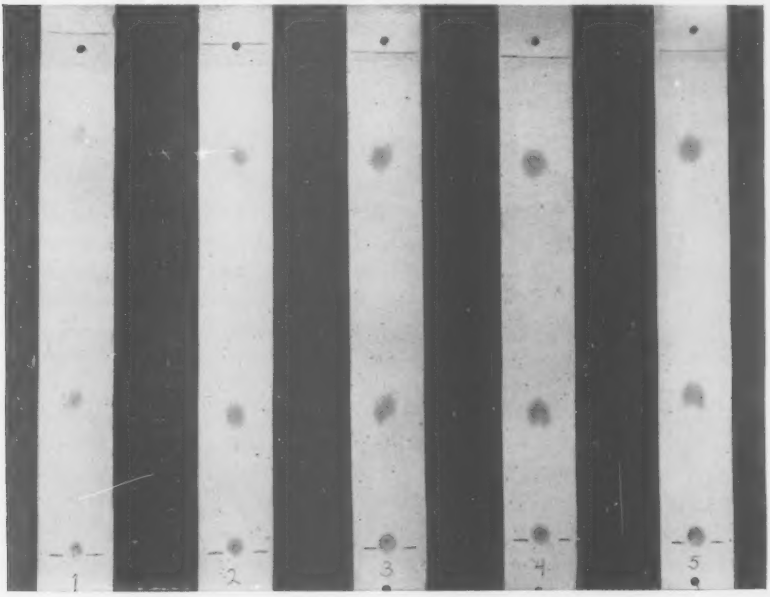


**Fig. 2.** Chromatogram of 1–5 μg. of estrone, estradiol-17 beta, and estriol.

### Elution

Elution of the colored estrogen spots was not feasible. The color sensitivity was attenuated in solution and fading was encountered with the several solvents tried. These solvents included ethyl and methyl alcohols, ethyl acetate, acetic acid, dioxane, and 6N sulfuric acid. The colored spots were not eluted with chlorinated hydrocarbons, benzene, and other nonpolar solvents.

### Humidity

Humidity was found to affect both the color stability and the fluorescence intensity of the estrogen spots. Color was found to fade when the chromatograms were exposed to room air for 1–3 hr. For this reason the strips were kept as dry as possible until analyzed. Fading was also encountered in strips left overnight over silica gel, although the fading was much less when kept protected in that fashion.

### Densitometry

Densitometry yielded valid results despite the lack of optimal conditions. The Analytrol, equipped with a B-5 cam and 500 mμ interference filters, imparts a curvilinear calibration to analyses of this sort. Estrogens measured at 500 mμ absorb only 83% of the peak absorption obtained at 525 mμ (Beckman DU). Therefore, it is obvious that sensitivity and linearity would be increased under more optimal conditions.

In the range of 1–5 μg. this procedure yields results that are definitive. Figure 3 shows the zone patterns of estrogens run as mixtures.

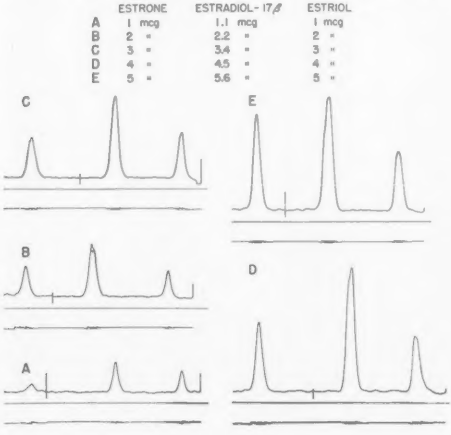


**Fig. 3.** Densitometrograms of several concentrations of estrogens. Starting point is on right. Vertical line between estrone (left) and estradiol-17 β is caused by manual movement of strip.

The peaks are sharp and symmetrical, tailing is absent, and smooth calibration curves can be drawn. All three estrogens show satisfactory sensitivity in this concentration range. Readings are not strictly reproducible between runs so that calibration controls are necessary for each chromatographic separation. One can also see that the readings in the higher concentration ranges are often suppressed.

The opacity of the glass paper requires that the zero adjust knob be rotated almost to the limit to obtain a zero point. However, with the lots of glass paper that were used in this study, attaining this setting was always possible. Figure 4 shows a typical calibration where the three estrogens were run in mixtures of varying concentration. The reason for the occasional leveling off at the higher concentrations is not known at this time.

### Fluorometry

The filter combination for the fluorometric method was chosen with the view of achieving suitable sensitivity while avoiding as much background fluorescence as possible.

The estrogens developed a pronounced fluorescence under the described conditions so that the surface concentration of the steroids allowed sufficient sensitivity to about 0.25 $\mu$g. This is indicated in Fig. 5, where concentrations (in micrograms) are plotted against dial read-

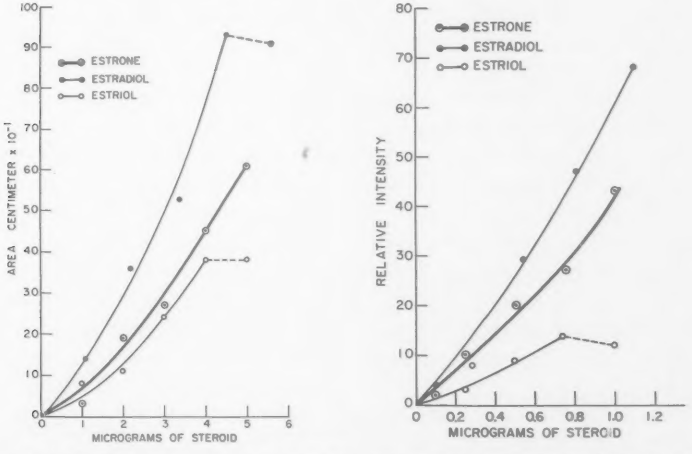


**Fig. 4** (*left*). Typical calibration curves for estrogen densitometrograms. **Fig. 5** (*right*). Typical calibration curves for chromatographic fluorescence scans.

ings (1X sensitivity). The calibration lines were usually curved and sometimes took on a sigmoid shape. Occasionally, estriol showed a shallow calibration curve but sufficient sensitivity was exhibited to allow quantitation. A 3X sensitivity may be used to provide readings of about two-fold those obtained with the 1X setting, but allowances must be made for a blank setting greater than zero.

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# Detection and Quantitative Determination of Glycine with Pyridine and *p*-Nitrobenzoyl Chloride

*Charles J. Umberger and Frank F. Fiorese*

**Glycine in quantities as small as 0.5 μg. can be detected as an orange-red color by a specific reaction with *p*-nitrobenzoyl chloride and pyridine. The procedure can be used both as a qualitative spot test and as a sensitive quantitative method. In a discussion of the reaction mechanism, it is suggested that the color is due to azlactone formation.**

SPECIFICITY in a qualitative test for glycine based on the color developed with pyridine and alkyl chloroformate was recently reported by Sublett and Jewell (*13*). Except for acetyl glycine and glycine hydrochloride, other amino acids and some other organic structures did not react in their method.

In our investigations of the reaction specificity for glycine, it was confirmed that, except for hippuric and salicyluric acids, which are indistinguishable from glycine at lower sensitivity levels, a variety of organic structures did not develop colors. However, except for specificity, the quantitative glycine test is lacking in the other requirements that are essential for a reliable quantitative reaction.

Interpretable colors were not obtained with less than 50 μg. of glycine, although the detectable limit was reported as 1–2 μg. Even at higher glycine concentration, the yellow color for the pyridine-ethyl chloroformate reaction is readily masked by the yellow color of protein hydrolysates and is obscured by urinary chromogens. With 100-mg. concentrations, the effervescence of the reaction within the white precipitate produced by the reagents alone disperses and dilutes the

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reddish glycine color within the solid sample. Color development is relatively slow, and the final color varies too widely between pale yellow and orange-red to permit semiquantitative evaluations. The failure of the color to diffuse away from the bulk of the precipitate in the paper spot test makes it difficult to evaluate the color in qualitative observations, particularly when colored impurities are present in the test sample. Heat generated on addition of the reagents makes the reaction at other than room temperature as reported.

Sublett and Jewell (*13*) showed that benzoyl chloride, acetyl chloride, thionyl chloride, and carbobenzoxy chloride do not react like ethyl chloroformate. However, we have found that *p*-nitrobenzoyl chloride not only reacts but is also far more sensitive and results in even greater specificity for glycine, with development at microgram levels of a red color which differs from normally occurring interferences. It is applicable as a qualitative spot test on paper or a glass slide and, unlike alkylchloroformates, immediately develops a stable color. In solution, it can serve as a quantitative color reaction for glycine.

## Qualitative Test

### Reagent

1. Solid *p*-nitrobenzoyl chloride recrystallized from petroleum ether
2. Pyridine (fraction distilling at 115°)

### Procedure and Results

A few crystals of unknown sample, rendered anhydrous, are placed on a filter paper strip or a glass slide, and approximately 1 mg. of solid *p*-nitrobenzoyl chloride is placed on top. One to three drops of pyridine are then added to wet the mixture. With glycine, an orange-red to maroon color develops immediately, varying in shade with the concentration of glycine. As little as 0.5 $\mu$g. of glycine develops a perceptible orange-red color.

All other amino acids fail to react in concentrations below 0.5 mg. With larger quantities, a pale bluish color, in contrast to colorless blanks, develops with some amino acids. Acetylglycine, glycylglycine, hippuric acid, and salicyluric acid do not react in the cold, but on warming, a yellow color is produced with milligram quantities.

The color is soluble in polar solvents such as chloroform, dichloroethylene, tetrachloroethane, ethylacetate, and also in excess pyridine.

If the procedure is carried out in a micro test tube, estimates of the

glycine content of the sample can be made by diluting to a predetermined color intensity.

Since water decreases the sensitivity, protein hydrolysates require dehydration, preferably by vacuum evaporation at room temperature.

Inorganic salts do not interfere, except for their dilution effect in increasing the bulk of solid sample.

The qualitative test is somewhat more sensitive when carried out on a glass slide, but it is more interpretable as a paper spot test because diffusion of the excess pyridine in the paper carries along and deposits some of the red color away from the mass of undissolved solid. This type of chromatographic migration of the color is particularly helpful when the unknown is a residue from urine or a sample from an acid hydrolysate of a protein.

## Color Test on a Paper Chromatogram

### Reagents

1. Saturated solution of *p*-nitrobenzoyl chloride in petroleum ether
2. Pyridine

### Procedure and Results

The dried paper chromatogram is sprayed with *p*-nitrobenzoyl chloride solution and allowed to air-dry. It is then sprayed with enough pyridine to dampen the paper strip. The color is red at the central area containing the glycine, changing to orange-red toward the outer area of the spot. The limit for detection is between 0.7 and 1.0 $\mu$g. of glycine after migration on a 1-in. paper strip.

## Quantitative Determination of Glycine

### Discussion of the Method

Some of the problems in transforming the test into a quantitative procedure are the insolubility of glycine in solvents other than water and the water insolubility and ease of hydrolysis of *p*-nitrobenzoyl chloride. The reaction is essentially nonaqueous, in that significant amounts of water prevent development of the color, while smaller amounts decrease the color intensity in proportion to the initial water concentration, regardless of the quantities of anhydrous reagents added later. The change in absorbance for 2 $\mu$g. of glycine mixed with increasing concentration of water in pyridine is shown in Fig. 1. When the water-to-pyridine ratio is 2:3, practically no color is developed, and

yet water is the only solvent that can be used for recovering glycine from test samples and for preparing glycine standards in the microgram concentration range.

It would seem possible to obtain anhydrous conditions through evap-

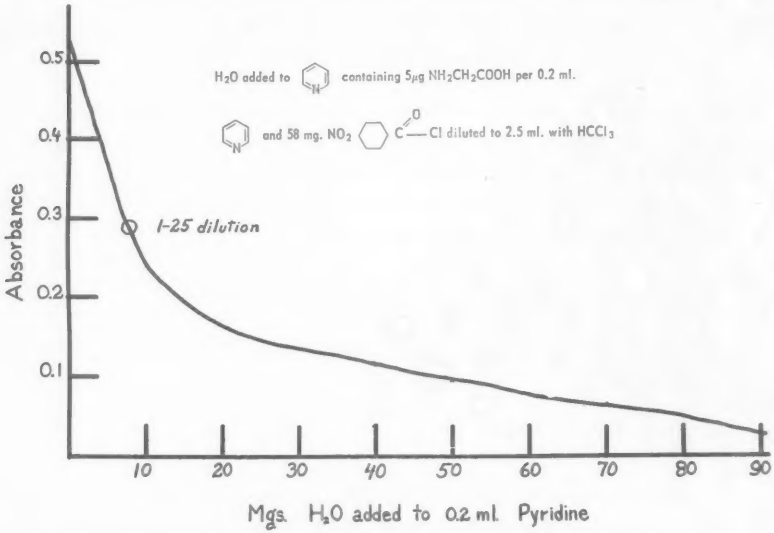



**Fig. 1.** Absorbance with increasing water at constant pyridine-*p*-nitrobenzoylchloride-glycine concentration.

oration of a measured volume of aqueous glycine solution to dryness in a cuvette, followed by addition of anhydrous pyridine and acyl chloride to the residue. Reproducibility is not possible with this process because the final color intensity is a function of the instantaneous concentration of *p*-nitrobenzoyl chloride, pyridine, and glycine. Glycine crystallizes on the sides of the cuvette during evaporation whereas the added solid *p*-nitrobenzoyl chloride remains on the bottom of the tube. Addition of pyridine results in a variable concentration ratio of the three components during development of the color. The critical conditions for color intensity are demonstrated in Fig. 2. Absorbance increases with increase in the *p*-nitrobenzoyl chloride content as the molar ratio approaches unity. Before the acyl chloride concentration reaches equivalent molar concentrations, the solution starts to develop an acid reaction to the Davidson (*4*) nonaqueous indicators, and from

this point on, the color decreases in intensity until, between ratios of 1:1 and 1:1.125, no color remains after dilution to volume with the solvent. Below the level for acidity and down to a ratio of 1:0.15, the solution is turbid and vigorous stirring is necessary during color de-

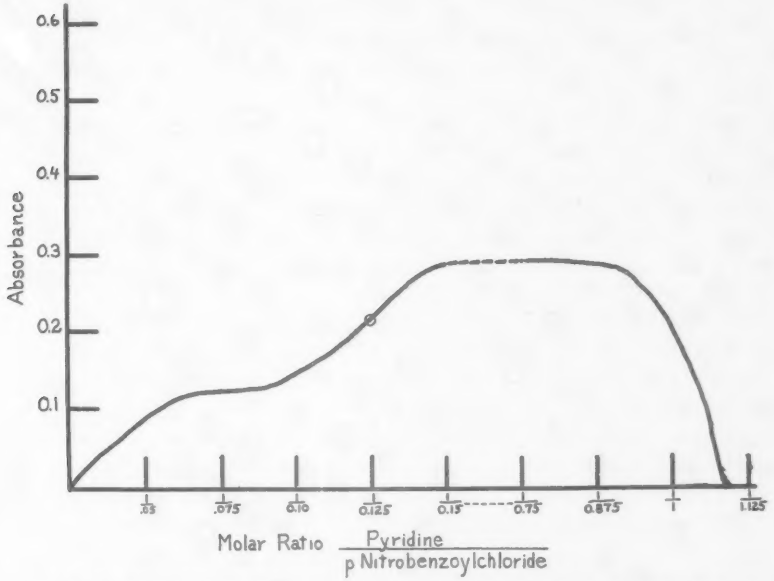



**Fig. 2.** Absorbance after dilution to 5 ml. with chloroform for 8.5 μg. of glycine in 0.4 ml. of 4% water-pyridine with increasing *p*-nitrobenzoylchloride concentration.

velopment because of the relatively slow rate of solution of the acyl chloride in pyridine. At a molar pyridine-to-*p*-nitrobenzoyl chloride ratio of 8, the absorbance is still significantly elevated, and since this lower acyl chloride content is readily dissolved with only slight agitation, 1M of pyridine to 0.125M of *p*-nitrobenzoyl chloride was selected as the best concentration for the quantitative reaction.

A freshly prepared reagent of *p*-nitrobenzoyl chloride dissolved in pyridine resulted in decreased and variable color intensities, and it became nonreactive after standing a few minutes. Other organic solvents for *p*-nitrobenzoyl chloride retard or prevent development of the color despite the fact that once formed, it readily dissolves without change in chloroform and related solvents.

As shown in Fig. 1, the absorbance decreases rapidly with slight increase in water content, leveling off to a more gradual change as the water concentration is further increased. The absorbance with 8 mg. of water in 0.2 ml. of pyridine is roughly 50% of that for the non-aqueous reaction, and yet the limiting sensitivity is still well below 5.0 μg. of glycine after dilution to 5 ml. with chloroform. This 4% (v/v) pyridine-water solution, which is a convenient 1:25 dilution ratio, was selected as the lowest practical limit in water content for the quantitative reaction. Since linearity requires a critical ratio for the reagents while water is essential for maintaining a homogeneous environment, the quantitative procedure represents a compromise in which a 1:25 water-to-pyridine ratio is employed while a molar pyridine-to-*p*-nitrobenzoyl chloride ratio of 1:0.125 is maintained. The upper concentration limit for the quantitative method is determined by the amount of glycine in water that will still remain in solution after dilution with 24 volumes of pyridine.

The solubility of glycine in water is given as 25.3 gm./100 ml. at 25° or 25.3 mg. in 0.1 ml. (*7*). The solubility in pyridine is given as 0.61 gm./100 ml., which is 14.6 mg./2.4 ml. This would suggest that precipitation should not occur on diluting 1 volume of aqueous glycine with 24 volumes of pyridine, provided the glycine was not prepared for analysis by taking it up in a volume to yield greater than a half-saturated aqueous solution. Moreover, the solubility data would suggest that anhydrous pyridine should serve as an ideal solvent as well as reactant for concentrations of glycine well above the microgram range. It was found that the pyridine solubility data are either in error or else the rate of solution for equilibrium is extremely slow. Digestion of glycine base or its hydrochloride with pyridine failed to yield solutions above 40 μg./100 ml. Immediate precipitation of glycine occurred upon addition of 24 volumes of pyridine to 1 volume of an aqueous glycine solution that contained more than 300 mg./100 ml. of the acid.

Although the 300 mg. of aqueous solution appeared clear when freshly diluted, glycine crystals deposited after standing overnight. Very dilute glycine solutions in 1:25 water-pyridine show a strong Tyndall effect, and high-speed centrifugation indicated that the solubility is not more than 2 μg. per milliliter. On dilution of 125 mg./100 ml. aqueous glycine solutions with pyridine, no measurable change in absorbance occurred during 4-hr. standing at room temperature. Aqueous glycine at this concentration and below forms a colloidal dispersion in 1:25 water-pyridine which is sufficiently stable to be treated

as a homogeneous solution within the period required for the quantitative determination. A 125 mg./100 ml. aqueous glycine solution is a suitable primary standard, and this level establishes the safe upper concentration limit for the aqueous extraction of glycine from an unknown sample. Beyond aqueous glycine concentrations of 3 mg. per milliliter, errors result through precipitation of glycine during the 1:25 dilution with pyridine.

## Procedure

### Reagents

1. Anhydrous pyridine
2. Chloroform
3. *p*-Nitrobenzoyl chloride washed with petroleum ether until free of *p*-nitrobenzoic acid

### Procedure and Results

In order that the final volume will not exceed 5 ml. of solution while keeping within the limited glycine concentration range, 0.4 ml. of 1:25 water in pyridine and 115.2 mg. of *p*-nitrobenzoyl chloride, with absorbance measured after dilution to volume with chloroform, were selected as optimum conditions for the quantitative method. The procedure is carried out by dilution of 0.1 ml. of aqueous glycine containing not more than 125 mg./100 ml. of glycine, to 2.5 ml. with pyridine. Larger volumes may be employed, maintaining a 1:25 dilution ratio. A 1:25 blank solution with distilled water is similarly prepared. Two 115.2-mg. accurately weighed samples of *p*-nitrobenzoyl chloride are introduced into 1-cm.-cell-depth cuvettes, 0.4 ml. of the 1:25 test and blank water-pyridine solutions are added, and the tubes are gently shaken to dissolve the acyl chloride. After standing about 2 min., the contents of the tubes are diluted to 5 ml. with chloroform and the absorbance of the test solution is measured against the blank at 480 m$\mu$. The absorption curve for glycine in anhydrous pyridine and in 1:25 water-pyridine is shown in Fig. 3. The decreased absorbance at the maximum and the washed-out bands in the presence of water are indicative of the nonaqueous character of the reaction. The absorbance as opposed to concentration curves shown in Fig. 4 demonstrate the critical conditions for maximum color development and reproducibility. The concentration range was not extended beyond 20 $\mu$g./5 ml. of solution because of error from pyridine precipitation of glycine. If the absorbance of an unknown aqueous extract indicates more than this

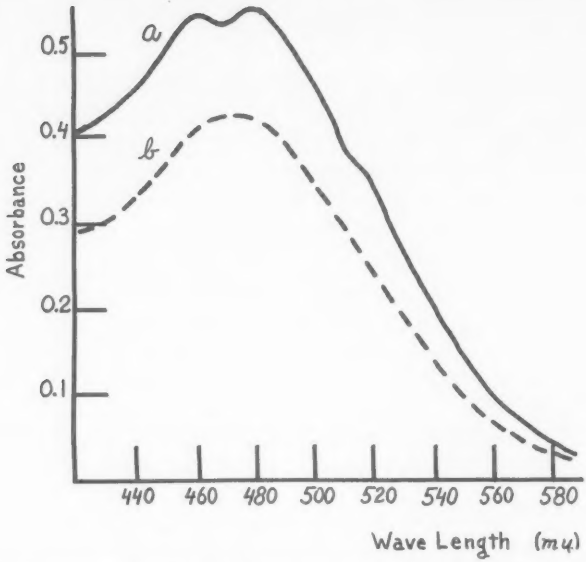



**Fig. 3.** Absorbance for glycine in anhydrous pyridine (Curve *a*) and in 1:25 water-pyridine (Curve *b*) with *p*-nitrobenzoylchloride diluted to 30 ml. with chloroform. Glycine concentration, 3.2 μg./ml. Molar ratio: Py:*p* NBC = 1:0.125.

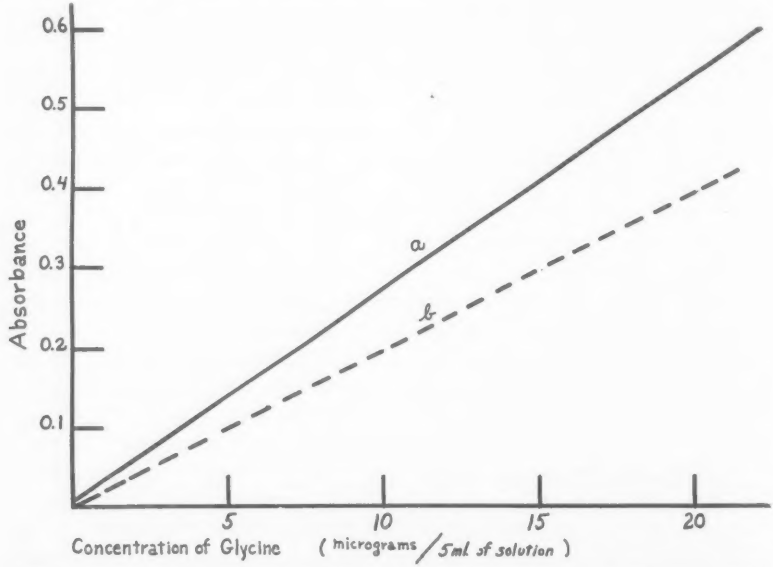



**Fig. 4.** Glycine concentration versus absorbance at 480 mμ (0.4 ml. of 1:25 water-pyridine with 115 mg. *p*-nitrobenzoylchloride diluted to 5 ml. with chloroform). Molar ratios: Curve *a:* Py:*p* NBC = 1:0.125; Curve *b:* Py:*p* NBC = 1:0.1.

glycine level, the original aqueous extract is diluted with water before making the 1:25 pyridine dilution.

## Studies of Reaction Mechanism

Solubility problems make studies of glycine and *p*-nitrobenzoyl chloride in pyridine difficult to interpret. The action of liquid benzenesulfonyl chloride and hippuric acid in pyridine is followed more readily, through development of the color. Findings indicated color from azlactone formation (*14*), with observations in agreement with the mechanisms proposed for the Erlenmeyer (*5*)–Plöchl (*11*) synthesis and the Dakin-West reaction (*3*). Evidence was obtained from the ultraviolet absorption that *p*-nitrobenzoyl chloride undergoes a similar condensation with hippuric acid in pyridine, with the difference that a much higher hippuric acid concentration is required and that the

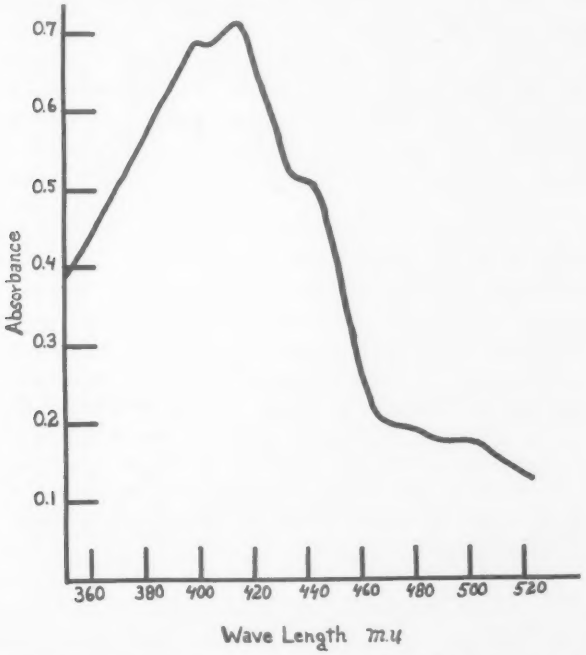
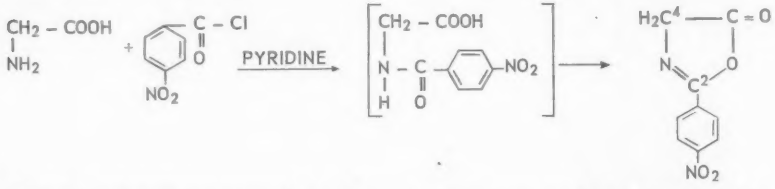



**Fig. 5.** Absorption curve for 2.5 mg. of glycine in 0.5 ml. of pyridine and 0.2 ml. of benzene sulfonyl chloride diluted to 5 ml. with chloroform. Molar ratio: Py:BSC = 4:1.

yellow color is not appreciably different from that of numerous organic condensations. The yellow colored solution produced on mixing milligram quantities of glycine in pyridine with benzenesulfonyl chloride also gives an azlactone type of ultraviolet curve (Fig. 5).

Similarities between the benzenesulfonyl chloride–pyridine reaction for hippuric acid and the *p*-nitrobenzoyl chloride–pyridine reaction for glycine indicate that color formation occurs as a result of the same mechanisms and suggest that both reactions are special cases of a more general type of azlactone formation, in which specificity in the color test arises from reaction of the compound with a particular condensing agent. This leads to the postulate that the ethyl chloroformate–pyridine test of Sublett and Jewell (*13*) for glycine is also azlactone formation in which the yellow product is insoluble in all of the common organic solvents, some of which, like water, destroy the colored complex. Application of the mechanism proposed for the Dakin-West reaction (*3*) to glycine requires conjugation at the amino nitrogen as the first phase of the cyclization:




Color development may also require conjugation at $C_4$, as discussed in the study of hippuric acid (*14*).

Despite the conformity of this mechanism with that proposed for azlactone formation by closely related amino acids, it is not entirely satisfactory for glycine. If it proceeds in the manner shown, *p*-nitrohippuric acid alone should give an azlactone in pyridine. All attempts at azlactone formation by treatment with pyridine failed to yield indications for an oxazolone. Treatment of a pyridine solution with benzenesulfonyl chloride and with acetic anhydride at higher concentrations of *p*-nitrohippuric acid resulted in the same behavior observed for hippuric and salicyluric acids.

The necessity for homogeneity, as found during the studies on reproducibility in the quantitative method, suggests that both pyridine and acyl halide play a more involved role than in the conventional conjugation reaction (*8*). Heat developed on dissolving *p*-nitrobenzoyl chloride in pyridine indicates addition but, after isolation of the prod-

uct of a reaction, the expected quaternary pyridine salt was not found. Isolation was accomplished by the drop-by-drop addition of pyridine to a petroleum ether solution of *p*-nitrobenzoyl chloride. The first drop of pyridine produced a cloudy solution, and with further addition, a dense white precipitate settled out. As more pyridine was added, the acidity measured with the Davidson nonaqueous indicators (*4*) decreased without further precipitation. When the precipitate was filtered and washed with petroleum ether until the washings no longer contained any residue after evaporation, a white crystalline solid remained.

Qualitative tests show a high chloride content, and cyanogen bromide with benzidine (*6*) develops an intense red color indicative of pyridine. The solid residue is strongly acid even when the acidity of the petroleum ether solution has been neutralized with excess pyridine. Quantitative evaluations of the pyridine content of the solid with cyanogen bromide and benzidine (*10*) showed that the molar pyridine content was slightly less than one-tenth of the *p*-nitrobenzoyl chloride, measured by the chloride content. All attempts to obtain a 1:1 ratio failed, and yet the derivative maintained constant composition in different preparations within measurable limits. Its melting point was 182–188°, and this elevation of 111° above the melting point for *p*-nitrobenzoyl chloride suggested a complex with more than 1M of the *p*-nitrobenzoyl chloride.

Freshly prepared precipitate mixed with glycine reacted slightly on grinding the solids but not when dissolved together in solvents; the reaction was undoubtedly hindered by the excess acidity of the precipitate. On the addition of a trace of pyridine to the solid mixture, an immediate color developed, confirming the excess of *p*-nitrobenzoyl chloride in the precipitate. After storage of the precipitate for 2 days in a stoppered bottle, the addition of pyridine and glycine to the precipitate no longer produced a color. In contrast to this behavior, solid *p*-nitrobenzoyl chloride still retained considerable activity with glycine and pyridine after standing exposed to air for 3 days.

When attempts at recrystallization from anhydrous solvents were made, and after storage in a vacuum desiccator the precipitate rapidly lost both chlorine and pyridine. Carbon, hydrogen, and nitrogen analysis* of the residue yielded the empirical formula $C_7H_5O_4N$, and the physical properties were indicative of *p*-nitrobenzoic acid. Despite the

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care taken to exclude moisture, degradation occurred far more readily than with *p*-nitrobenzoyl chloride, possibly through a type of auto-oxidation. Acyl halide rupture of the pyridine ring to form a glutaconic aldehyde (*6*), as occurs in the cyanogen bromide generic test for the pyridine nucleus unsubstituted in the alpha position, was ruled out after failure to obtain a color with either benzidine (*9*) or thiobarbituric acid (*12*).

It would appear that the function of the pyridine is more that of reagent than catalytic base, as has been suggested (*1*, *2*), as its influence on the acyl halide is more involved than merely enhancing condensation. The mechanism leading to the intermediate form in the cyclization apparently requires further elucidation for an understanding of the azlactone mechanism.

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# Colorimetric Method for Hippuric Acid

*Charles J. Umberger and Frank F. Fiorese*

**Hippuric acid dissolved in pyridine produces a red-orange color upon the addition of benzenesulfonyl chloride. The color has a stable absorbance at 380 mμ. It is sensitive in microgram quantities of hippuric acid and follows the Beer-Lambert Law over a wide range of concentrations. The mechanism of the color reaction is described.**

HIPPURIC ACID is an important metabolic product normally present in urine in amounts determined by the ingestion of benzyl-containing foods and medicinals (*11*). Because this compound is comparatively inert chemically, identification requires isolation and the determination of physical constants after extensive purification. There has long been need for a reliable and sensitive color reaction for its detection and for estimation of low-level concentrations.

In attempts to enhance chromatographic separations of components in acidic extracts of urine through preparation of benzoyl and related derivatives, development of a deep-red color with benzenesulfonyl chloride (BSC) in the presence of pyridine (Py) was traced to hippuric acid (HA) in the mixture. Studies of the reaction led to a sensitive color test applicable for quantitative measurements. Other alkyl and aryl acid halides failed to develop significant colors and amino acids and related acidic compounds did not react in the test. Salicyluric acid is equally sensitive and probably other amido acids are a source of interference. Confirmation of HA is readily made following positive findings for an amido structure in the test.

## Qualitative Test

A drop of Py is added to a crystal of anhydrous solid sample contained in a micro test tube, and mixed to dissolve the solid. An equal

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volume of BSC is layered on top. The pressure of HA produces a deep-red ring at the interface and, on mixing, the solution develops an orange-red to deep-red color determined by the concentration. A detectable color is obtained with 0.3 μg. of HA in two drops of test solution.

### Quantitative Reaction

The absorption curve for the red solution in chloroform is shown in Fig. 1. A maximum in the curve occurs at 420 mμ when the measurement is made within 3 min. On further standing the 420 maximum gradually decreases, with a change in color from red to orange. The absorbance maximum then shifts toward 380 mμ. Since no further change in absorbance occurs at 380 mμ, this wave length was selected for the quantitative measurement.

Studies on the absorbance developed with different amounts of hippuric acid in varying molar ratios of pyridine and BSC showed that maximum color development occurs with a ratio of 4 moles of Py to one mole of BSC. Increasing the Py decreases the color intensity. With an increase in BSC the decrease in color is more pronounced. With equimolar concentrations, the solution shows an acid reaction to the Davidson nonaqueous indicators (*5*) and, as acidity increases, color development becomes progressively less (Fig. 1).

The order of addition of the reagents has a marked influence on the color development. Hippuric acid added to BSC chloride and treated with Py does not develop as intense a color at a given HA level and it fades more rapidly to an orange. Hippuric acid added to a freshly prepared solution of pyridine and BSC develops still less color with a marked drop in absorbance at 420 mμ, below the value found at 380 mμ (Fig. 2).

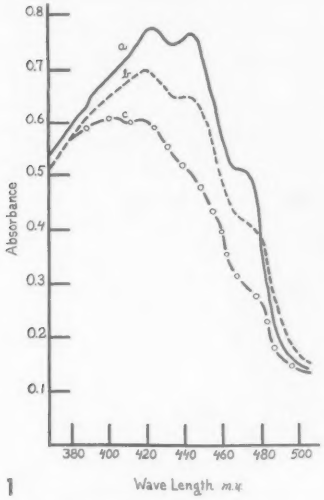
### Procedure

A quantity of sample is dissolved in a volume of Py to contain between 0.5 and 135 μg of HA per 0.5 ml. of solution. The 0.5 ml. of Py solution is introduced into a cuvette, 0.2 ml. of BSC added, and the solution mixed and allowed to stand 20 min. or longer. It is then diluted to 5 ml. with chloroform and read at 380 mμ against a comparable Py-BSC blank. The Beer-Lambert curve for 1-cm. cell path is shown in Fig. 3.

### Mechanism of the Reaction

Pyridine is frequently employed in benzoylation and similar condensation reactions which require removal of halogen acids, especially

when saponification of the ester group can occur through use of alkali. Salt formation between acid and tertiary amine is not limited to strong inorganic acids. Addition compounds with acidic organic structures have been isolated (*6*), and the immediate development of heat on dissolving acyl halide in pyridine indicates that additive compounds are generally formed (*10*).

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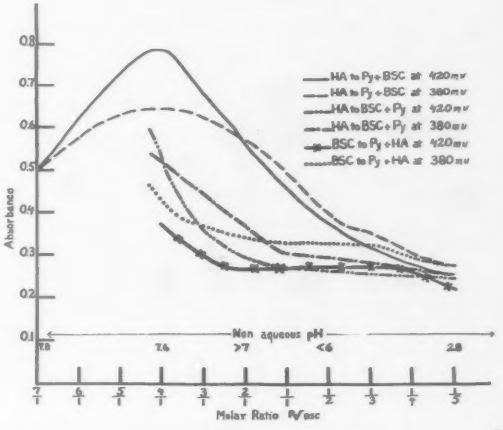


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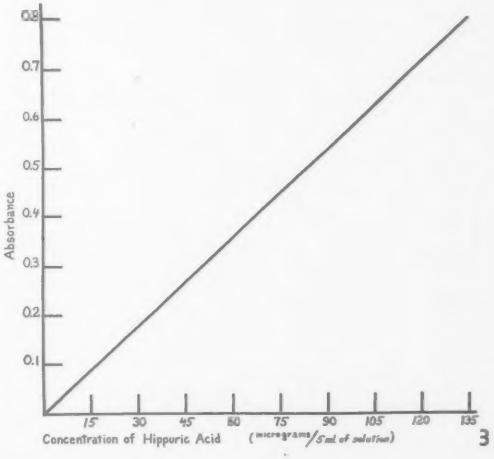
**Fig. 1.** Absorbance for HA in Py and BSC. Curve *a* indicates that for molar ratio Py: BSC = 4:1, with 20 μg. HA in 0.5 ml. Py and 0.2 ml. BSC diluted to 5 ml. with chloroform; *b*, molar ratio Py:BSC = 5:1; and *c*, the same as curve *b* after standing 20 min. **Fig. 2.** Variation in absorbance at 380 and 420 mμ for 20 μg. of HA with different ratios of Py to BSC and with the order of addition of the reagents as shown for the various curves. Molar ratio of 4:1 equals 0.5 ml. Py to 0.2 ml. BSC diluted to 5 ml. with chloroform. **Fig. 3.** Absorbance vs. HA concentration at 380 mμ (0.5 ml. Py and 0.2 ml. BSC diluted to 5 ml. with chloroform).

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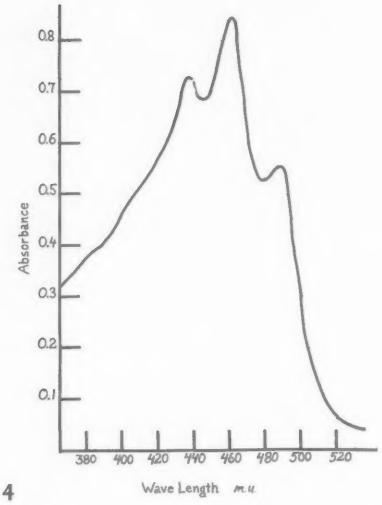
The decrease in color intensity on adding HA to a Py-BSC solution indicates that compound formation between the reagents is not involved in the reaction. Similarly, the decrease in color on adding Py to a HA-BSC solution suggests that condensation at the amido nitrogen is not a requirement for color development.

The shape of the ultraviolet absorption curve (*9*) and the importance of maintaining a basic environment suggests that the color results from an azlactone, formed through a mechanism similar to that in

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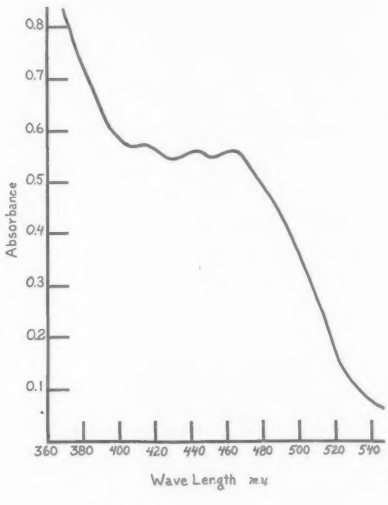


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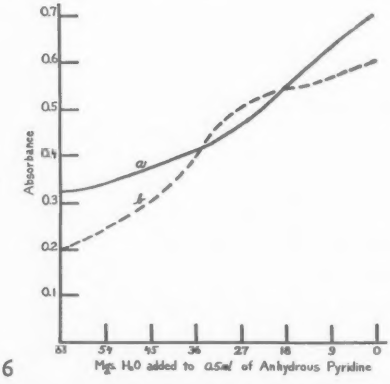


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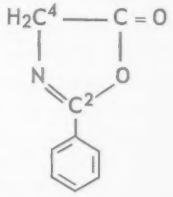
**Fig. 4.** Absorbance for 1.5 mg. HA in 1 ml. Py and 0.3 ml. acetic anhydride diluted to 15 ml. with chloroform. **Fig. 5.** Absorbance for 1 mg. HA in 0.4 ml. Py with 115 mg. *p*-nitrobenzoyl chloride diluted to 30 ml. with chloroform. **Fig. 6.** Influence of $H_2O$ on absorbance at 380 and 420 mμ for 100 μg. HA in 0.5 ml. Py and 0.2 ml. BSC diluted to 5 ml. with chloroform. Molar ratio Py:BSC = 4:1. Curve *a*, 420 mμ; *b*, 380 mμ.

the Erlenmeyer (*7*)-Plöchl (*8*) azlactone synthesis. Condensation of HA with *p*-fluorobenzaldehyde in the presence of acetic anhydride and anhydrous sodium acetate in this synthesis yields a mixture of 2-phenyl-and 2-methyl-4-(*p*-fluorobenzal-5-oxazolones) (*1*). The gradual disappearance on standing (Fig. 1) of the 420 mμ absorption can be explained by the two pathways for the color reaction, with one of the forms consisting of a metastable compound and the other a stable structure. The mechanism for the Dakin-West reaction (*4*) of alpha-amino acids with acetic anhydride in the presence of pyridine is believed to involve a base-catalyzed acylation which is similar to that in the Erlenmeyer-Plöchl azlactone formation (*2, 3*).

In studies on the behavior of HA in Py after addition of acetic anhydride, it was observed that an orange-red color, similar to that with BSC, developed when milligram quantities of hippuric acid were used. Conditions for color development with acetic anhydride were the same as those required for BSC, indicating that the two reactions are similar except that the one is sensitive in the microgram range, while the other requires milligram quantities and at least 40 min. for full color development. The ultraviolet absorption curve (Fig. 4) has the three maxima suggestive of an azlactone and closely resembles that for the BSC compound (Fig. 1).

It is reasonable that acyl chlorides, which are anhydrides of an organic and inorganic acid, can substitute for anhydrides in the Dakin-West reaction, and analogy with the proposed mechanisms would suggest that color formation with an oxazolone results from acylation at the $C_4$ methylene carbon and possible transacylation at $C_2$, as demonstrated by Bennett & Niemann (*1*).




The work of Schueler & Wang (*9*) suggests that development of color and color sensitivity would depend on substitution at the $C_4$, with the chromatic nature of the oxazolone molecule determined by the particular acyl radical employed.

Other acyl halides failed to develop significant colors except for *p*-

nitrobenzoyl chloride, which develops a relatively insensitive and unimpressive yellow but which shows absorption suggestive of an azlactone (Fig. 5). The nonaqueous character of the reaction shown in Fig. 6 indicates that water influences the products of reaction as well as inhibiting color formation.

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# Spinal-Fluid Protein Determination for the Differentiation of Neurologic Disorders

*N. M. Papadopoulos,* W. C. Hess, D. O'Doherty, and L. Wakeman*

**A simple chemical test previously described for the determination of total protein and γ-globulin was applied to the analysis of spinal fluid specimens (CSF) from non-neurologic and a variety of neurologic disorders. On the basis of the chemical findings, the pathologic specimens analyzed were divided into three groups: (a) non-neurologic and some neurologic conditions with a normal CSF total protein and γ-globulin concentration; (b) demyelinating disorders with a normal total protein and elevated γ-globulin concentration; and (c) degenerative and infectious with an elevated total protein and γ-globulin. A good correlation between chemical findings and clinical observation was obtained.**

NEUROLOGIC DISEASES of demyelinating, degenerative, inflammatory, infectious, and toxic origin have a number of symptoms in common. The task of the neurologist to differentiate these conditions on the basis of clinical observations alone is often difficult, and constant research is being made for helpful laboratory tests. Proteins are present in cerebrospinal fluid (CSF) in measurable amounts, and an alteration of their pattern and concentration has frequently been associated with neurologic diseases. A number of investigators determined proteins in spinal fluid by electrophoretic, immunochemical, and chemical methods during studies of multiple sclerosis (MS) and other neurologic disorders (*1–5*). Our efforts have been directed toward establishing a simple chemical test for the differentiation of certain neurologic diseases.

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In a previous communication a procedure was described for the determination of total protein and $\gamma$-globulin in spinal fluid (*6*). The present paper gives the results of the application of the method to the analysis of spinal-fluid samples from patients with various neurologic disorders and a comparison of the chemical findings with the clinical diagnosis.

## Experimental

The following details should be observed in the procedure previously described (*6*) in order to obtain reproducible results.

1. The amount of zinc sulfate in the barbital buffer should be 1.45 gm., instead of 1.75 as previously reported. If the mixture of barbital and sodium barbital does not result in a *p*H 7.3, the buffer is adjusted to a final *p*H 7.3 with NaOH. This buffer is kept refrigerated, and is used cold for the precipitation of $\gamma$-globulin in spinal fluid. After centrifugation, the precipitated $\gamma$-globulin is washed once with 4 ml. zinc sulfate buffer diluted with water 1:1 v/v.

2. The phenol reagent is added into the mixture dropwise by means of a 10-ml. biuret and simultaneous stirring by a vortex mixer.* The phenol reagent is rapidly destroyed in the alkaline conditions of the mixture, and the gradual addition allowed the formation of a good color and gives consistent results.

## Results and Discussion

The zinc sulfate precipitate is called $\gamma$-globulin in this paper in order to be consistent with previous publications. It is possible, however, that the zinc sulfate precipitate consists of different types of $\gamma$-globulins; it has also been reported recently by Hass and Hochwald (*7*) that it contains other protein fractions which may have escaped detection by previously used technics. Examination of the zinc sulfate protein precipitate is presently under investigation in our laboratory, using column chromatography, and the results will be reported elsewhere.

Lumbar spinal fluid was centrifuged and analyzed within 24 hr. after collection. All samples which contained blood were discarded. The chemical analysis was done independently of the clinical studies. After completion of the laboratory studies, the final diagnosis was made on clinical grounds.

On the basis of the chemical findings, the specimens analyzed were divided into three groups, as shown in Table 1: (a) those in which the

*Scientific Industries, Inc., Springfield, Mass.

**Table 1.** SPINAL-FLUID TOTAL PROTEIN (TP) AND GAMMA GLOBULIN ($\gamma$-G) (CONCENTRATION MG./100 ML.)

| Diagnosis | No. of cases | TP Mean and S.D. | TP Range | $\gamma$-G Mean and S.D. | $\gamma$-G Range | $\gamma$-G, % of TP Mean and S.D. | $\gamma$-G, % of TP Range |
|---|---|---|---|---|---|---|---|
| I. Normal | | | | | | | |
| 1. Normal controls | 12 | 35 ± 3.0 | 25-45 | 2.5 ± 0.7 | 1-3 | 7 ± 1.8 | 4-9 |
| 2. Non-neurologic conditions | 16 | 36 ± 3.1 | 26-46 | 2.7 ± 0.8 | 1-3 | 8 ± 2.0 | 4-9 |
| 3. Neurologic conditions | | | | | | | |
| a. Neuritis and myelitis | 10 | 33 ± 2.8 | 27-43 | 2.3 ± 0.6 | 1-3 | 7 ± 2.2 | 3-9 |
| b. Heredofamilial degeneration | 7 | 40 ± 2.5 | 29-46 | 2.8 ± 0.7 | 2-3 | 7 ± 2.0 | 3-8 |
| c. Psychiatric | 18 | 42 ± 3.0 | 30-47 | 3.0 ± 0.9 | 2-4 | 7 ± 2.3 | 4-9 |
| d. Alcoholism | 8 | 40 ± 2.8 | 28-44 | 2.5 ± 0.8 | 1-3 | 6 ± 2.5 | 3-9 |
| II. Demyelinating | | | | | | | |
| 1. Multiple sclerosis | 40 | 43 ± 8.2 | 27-61 | 11 ± 2.3 | 6-16 | 25 ± 5.0 | 16-35 |
| 2. Retrobulbar neuritis | 4 | 35 ± 2.8 | 22-39 | 4 ± 1.0 | 4-6 | 11 ± 2.3 | 9-18 |
| 3. Encephalomyelopathy | 8 | 37 ± 3.0 | 32-46 | 5 ± 1.2 | 4-6 | 13 ± 1.8 | 11-15 |
| III. Degenerative and infectious | | | | | | | |
| 1. Polio | 2 | 125 | | 20 | | 16 | |
| 2. Cerebrovascular lesions | 4 | 50 ± 2.5 | 48-54 | 6 ± 2.0 | 4-10 | 12 ± 3.0 | 8-16 |
| 3. Cerebral atrophies | 4 | 48 ± 5.0 | 38-50 | 7 ± 3.0 | 4-12 | 14 ± 4.0 | 10-20 |
| 4. Brain tumors | 6 | 75 ± 35.0 | 45-150 | 11 ± 7.0 | 5-27 | 14 ± 6.0 | 8-24 |

total protein and $\gamma$-globulin were normal, as in the controls, (b) those with a normal total protein and an elevated $\gamma$-globulin, and (c) those with elevated total protein and $\gamma$-globulin.

Normal controls were specimens obtained from spinal anaesthesia. Non-neurologic conditions included samples which came to our laboratory because a neurologic disorder was initially suspected and which proved later by both chemical and clinical findings to be non-neurologic. Most of the conditions belonging in this group were: eye and ear diseases (diplopia, central retinal thrombosis, vertigo, Meniere's syndrome).

The neurologic conditions included: (a) peripheral nerve (neuritis) and spinal cord (myelitis)—in these conditions, the CSF protein con-

centration was normal; (b) the heredofamilial degenerative conditions, which by nomenclature and neurologic classification belong in the third group of degenerative conditions—interestingly, these have a normal CSF protein content; (c) most of the psychiatric cases which did not involve organic disturbance of the brain—these gave normal values and were differentiated from the few which were associated with other neurologic conditions; (d) acute alcoholism which apparently was not the result of structural changes of the brain—this belongs in the same group with the normal values.

Demyelinating conditions are characterized chemically by a normal total protein and an elevated $\gamma$-globulin. Clinically, the differentiation of the various demyelinating conditions is difficult. The data show that the ratio ($\gamma$-globulin/ total protein) in MS is higher and characteristically different from the other demyelinating conditions studied. These findings, although not conclusive, because of the limited number of cases studied, are consistent with the belief that retrobulbar neuritis is an initial episode of MS.

The 40 MS cases shown in Table 1 represent only 75% of all the cases analyzed in which the chemical findings are in agreement with the clinical diagnosis. In 10 cases with a clinical diagnosis of MS, the chemical values were normal. A closer examination of those cases revealed that in 4 of the 10 cases, the CSF was analyzed after the patients had received treatment with steroids. In 2 of these cases the decrease of the CSF $\gamma$-globulin level after steroid treatment was associated with an improvement in their clinical condition. These preliminary findings indicate that some MS patients may respond to steroid therapy. In 3 other MS cases, with normal chemical values, the clinical diagnosis was not definitive.

In the third group, elevation of total protein is associated with $\gamma$-globulin elevation. The data show that the $\gamma$-globulin/total protein ratio of this group is different from the first group and MS, but similar to the other demyelinating conditions. They are differentiated from the latter by their high total protein.

Poliomyelitis, a virus infection, affects the anterior horn cells of the cord and brain stem. The cerebrovascular lesions include cerebral arteriosclerosis and thrombosis. These represent half of all the cases studied having moderately elevated total protein and $\gamma$-globulin; the remainder gave normal values. The same was found in the cerebral atrophies. It is possible that only severe cases of cerebrovascular lesions and cerebral atrophies are reflected in CSF by an elevation of

total protein and $\gamma$-globulin. Six brain tumor-cases were found to have an elevated CSF total protein and $\gamma$-globulin content. Two brain tumor cases gave normal values and have not been included.

It is concluded that chemical analysis of CSF proteins provides additional information for the differentiation and diagnosis of neurologic diseases. These preliminary laboratory findings and their clinical application give impetus to further studies on a larger number and variety of neurologic disorders.

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# A New Simple Semimicro Method for Colorimetric Determination of Urea

*J. J. Coulombe and L. Favreau*

**A new simple and rapid method for quantitative semimicro determination of blood urea is presented. Diacetyl monoxime and thiosemicarbazide form in acid medium with urea a red color complex with a maximum adsorption at 530 mμ stable at room temperature and requiring 0.2 ml. of 1:10 blood filtrate.**

THE GREAT MAJORITY of the direct methods for the determination of urea in biologic fluids (*2–16*) are based on the Fearon's reaction: the formation of a alpha-diketone-urea complex (*1*). The two diketones used are the diacetyl (butanedione 2-3) and the acetyl benzoyl (1-phenyl propane dione 1-3) with a preference for the former in the monoxime form. It is to be noted that the reaction is photosensible (*7, 9, 10*), does not follow Beer's law, (*2, 7, 9, 11*) and requires a mixture of strong acid and oxidizing agent the nature and concentration of which are critical (*5, 9, 11, 15, 16*).

In 1948, Wheatley (*6*) used phenylanthranilic acid as an intensifier, for the reaction between diacetyl monoxime and urea. Effectively the sensitivity of the reaction was increased, but unfortunately the effect of light was more marked. The very low solubility of phenylanthranilic acid in water was a cause of trouble.

In 1961 Beale and Croft (*16*) studied in much detail the different factors involved in the Wheatley technic. They tried without success to replace the phenylanthranilic acid by another aromatic secondary amine and finally they proposed a new acid mixture which contained chloride ions to activate the reaction, phosphate ions to increase the reproducibility, and manganese ions to decrease the photosensitivity.

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We believe that we simplify the Beale and Croft technic by replacing phenylanthranilic acid by thiosemicarbazide, which is soluble in water and gives a nonphotosensitive complex with diacetyl monoxime and urea. We also substitute for the acid mixture of the authors, a simple dilution of phosphoric acid.

## Method

### Reagents

1. *Sodium tungstate, 10% w/v* Dissolve 10 gm. of sodium tungstate, C.P., in distilled water and dilute to 100 ml. Neutralize the solution with 1N sulfuric acid using phenolphtalein as indicator.

2. *Sulfuric acid N/12* Prepared by mixing 2.32 cc. of conc. $H_2SO_4$, C.P., with distilled water and diluting to 1000 ml. Normality should be checked.

3. *Tungstic acid** Mix just before use, 9 parts of N/12 sulfuric acid (*2*) with 1 part of sodium tungstate 10% (*1*).

4. *Phosphoric acid 60% v/v* Mix 60 cc. of phosphoric acid, C.P., (85.0–87.0%) with distilled water and dilute to 100 ml. This solution is stable indefinitely.

5. *Diacetyl monoxime-thiosemicarbazide (DAM-TSC)* Dissolve 0.6 gm. of DAM (2, 3, butanedione-2-monoxime, C.P.) and 0.03 gm. of TSC, C.P., with distilled water and dilute to 100 ml. This solution is stable at room temperature. A pale yellow color which appears after few days does not interfere with the reaction.

6. *Reagent A* Just before use, mix 10 parts of phosphoric acid 60% (*4*) and 2 parts of DAM-TSC (*5*). This solution is not stable for more than 1 hr.

7. *Urea Standard solution, 150 mg./100 ml.* Dissolve 150 mg. of urea, C.P., with distilled water and dilute to 100 ml. Add 1 drop of preservative (benzalkonium chloride 12.8%†) per ml. if the solution is to be kept. Prepare various diluted solutions from the stock standard and use like blood specimen to calibrate the spectrophotometer.

### Procedure for Blood Specimens

Collect 0.1 cc. of blood obtained from finger tip puncture with 0.1 cc. pipet and add immediately to a small centrifuge tube containing 0.9 cc. of tungstic acid (Reagent 3). After 5 minutes centrifuge and collect

*Stable tungstic acid (Hycel), Scientific Products Co., Div. of American Hospital Supply Corp., Evanston, Ill.

†Zephiran, Winthrop Laboratories, New York, N. Y.

0.2 ml. of supernatant liquid with a 0.2-ml. pipet, add to 5 ml. of Reagent A (No. 6) in a test tube. After vigorous shaking, heat in a boiling water bath for 20 min. ± 1 min. Remove immediately and cool in running water to room temperature. Read immediately in a spectrophotometer at 530 mμ or in a colorimeter using a yellow-green filter, adjusting the 100% T with a blank made with Reagent A.

### Procedure for Urine

The urine is diluted 1:20 with water and the analysis is made as for blood specimens. The concentration is calculated from the calibration charts for blood which is then multiplied by the dilution factor.

## Acid Variations

It is necessary to use acid ions to produce a colored complex with DAM-TSC. Sulfuric acid, mixtures of sulfuric-hydrochloric acid, and phosphoric acid were studied and the latter was selected for reasons of simplicity in preparing the reagents.

Sulfuric acid alone produces an increasing sensitivity for the reaction up to an optimum acid concentration of 25–30%. Higher acid concentration destroys the colored complex. A mixture of sulfuric acid, 30%, and hydrochloric acid, 1.5–2%, greatly improves the linearity of the color produced. The absorbance of the blank is also increased with the addition of hydrochloric acid. Traces of nitrates also greatly improved the reaction, but the reaction is nonlinear at urea concentrations of 100 mg./100 ml. and above. The addition of a phosphate buffer did not seem to have any effect on the reaction. It is of interest to note that the acid mixture of Beale and Croft (*16*), used for urea-phenyl anthranilic acid complex formation, also produces with urea–DAM-TSC, a very sensitive and linear reaction. Commercial phosphoric acid, 85–87%, C.P., is used as acidifier at an optimum final concentration of 45–55% for urea concentration in the 10–150 mg./100 ml. range. The great stability of the reagent and the ease of handling at such dilution make its use practical even if reaction sensitivity is decreased about 20–35%.

### Complexing Reagents Variations

A large number of secondary amines were assayed to produce a colored complex with diacetyl monoxime and urea. We selected TSC for various reasons, mostly solubility, stability, and ease of obtaining

the purified product. Various final concentrations of DAM, from 0–1000 mg./100 ml., gave increasing color production with increasing colored blank. A 100 mg./100 ml. concentration gave good results and relatively low blank (absorbance 0.03 and less). A large selection of TSC concentration was also studied. Up to a final concentration of 10 mg./100 ml. the reaction kept its linearity and sensitivity, but higher concentration, up to 400 mg./100 ml. failed to produce any improvement. The final concentration of DAM (9.89 mM, or 0.1%) and TSC (0.549 mM, or 5 mg./100 ml.) is the mixture of choice.

### Heating Time at 100°

All the reactions were studied at 100° (boiling water bath) and no efforts were made to change the temperature. No specific end of reaction is noted after a shortened heating period. After 15–18 min. a marked decrease in speed of reaction was noted. At 20 min. heating and then cooling immediately in running water to room temperature, the color is maximum and stable for many hours. Following the proposed procedure, easily reproducible results were obtained, as seen in Table 1.

Using the Henry and Segalove method (± 2.65 R) and a series of 85 duplicate analyses of routine unknown specimens, the confidence limits obtained with the proposed technic were ± 2.16 mg./100 ml.

### Spectrum

The absorbance spectrum of the urea reaction product has a broad band in the visible range with max. 535 mμ. The spectrum was measured with a Beckman DU spectrophotometer and a graphic recorder (Fig. 1). Any colorimeter with filter between 500 and 550 mμ is satisfactory. The following instruments were used for our studies: Beck-

**Table 1.** Stability of the Color Produced at Room Temperature

| | *Absorbance* | | | | | |
|---|---|---|---|---|---|---|
| | *Time after heating and cooling completed* | | | | | |
| *Urea (mg./100 ml.)* | *0 min.* | *15 min.* | *30 min.* | *45 min.* | *60 min.* | *120 min.* |
| 30 | .090 | .091 | .090 | .090 | .090 | .091 |
| 60 | .180 | .178 | .178 | .175 | .177 | .178 |
| 90 | .267 | .263 | .265 | .267 | .261 | .265 |
| 120 | .367 | .354 | .354 | .349 | .352 | .354 |
| 150 | .444 | .444 | .438 | .435 | .438 | .441 |

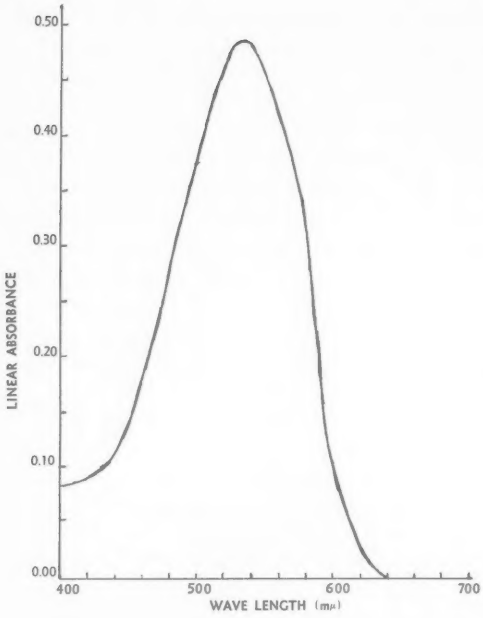


**Fig. 1.** Absorbance spectrum of urea reaction product.

man DU spectrophotometer,* Coleman Universal spectrophotometer,† and Leitz photometer‡ for routine purposes.

### Comparison of Present Method with a Diacetyl Method

Comparisons between the proposed procedure and the usual Rosenthal method were made over a period of 4 months. All the results presented in Table 2 are the average of duplicate determinations and were obtained under normal daily routine conditions. The average difference was ± 2.3%. No difficulties were encountered for urea determination in urine diluted 1:20. The possible interference of some naturally occurring substances was assayed. A 100 mg./100 ml. concentration of each of the following substances was assayed: creatinine, creatine, uric acid, ammonia, and 20 different amino acids. In no instances did any of these products give a color formation with DAM-TSC in the conditions of analysis.

*Beckman Instruments Inc., Spinco Division, Palo Alto, Calif.
†Coleman Instruments, Inc., Maywood, Ill.
‡E. Leitz, Inc., New York, N. Y.

**Table 2.** Comparison of the Present Method with One of Diacetyl Alone

| Specimen No. | Urea (mg./100 ml.) DAM-TSC | Urea (mg./100 ml.) DAM | % difference (DAM-TSC)−(DAM) |
|---|---|---|---|
| 1 | 19.5 | 20 | −2.5 |
| 2 | 30.5 | 29 | +5.0 |
| 3 | 24.5 | 25 | −2.0 |
| 4 | 23.5 | 23 | +2.1 |
| 5 | 27 | 26 | +3.7 |
| 6 | 19.5 | 20 | −2.5 |
| 7 | 21 | 20 | +5.0 |
| 8 | 48.5 | 50 | −3.0 |
| 9 | 34 | 33 | +2.9 |
| 10 | 36 | 35 | +2.8 |
| 11 | 132 | 128 | +3.1 |
| 12 | 28 | 30 | −7.1 |
| 13 | 44 | 46 | −4.3 |
| 14 | 42 | 42.5 | −0.9 |
| 15 | 33 | 34.5 | −4.5 |
| 16 | 51 | 49 | +4.0 |
| 17 | 112 | 110 | +1.8 |
| 18 | 32 | 31 | +3.2 |
| 19 | 44 | 47 | −6.8 |
| 20 | 51.5 | 50 | +2.9 |
| 21 | 90.5 | 92 | −1.7 |
| 22 | 31 | 33 | −6.3 |
| 23 | 37 | 38 | −2.7 |
| 24 | 57 | 56 | +1.8 |

Average % difference ± 2.5%.

**Table 3.** Reproducibility of Determination

| Reagent batch No. | Urea (mg./100 ml.) | Absorbance | Mean reading | Reading as % mean |
|---|---|---|---|---|
| | 54 | .167 | | 100.0 |
| 136 | 57 | .173 | .167 | 103.0 |
| | 54 | .167 | | 100.0 |
| | 52 | .161 | | 96.5 |
| | 48 | .148 | | 100.0 |
| 115 | 47 | .145 | .148 | 98.0 |
| | 50 | .154 | | 104.0 |
| | 48 | .148 | | 100.0 |
| | 33 | .105 | | 105.0 |
| 20 | 32 | .102 | .100 | 102.0 |
| | 31 | .099 | | 99.0 |
| | 30 | .096 | | 96.0 |
| | 118 | .359 | | 87.0 |
| 50 | 124 | .374 | .368 | 109.0 |
| | 120 | .367 | | 99.0 |
| | 122 | .372 | | 105.0 |

Table 4. Stability of Reagents

| *Urea (mg./100 ml.)* | *Time reagents kept at room temperature* | | | |
|---|---|---|---|---|
| | *1 wk.* | *5 wk.* | *10 wk.* | *20 wk.* |
| 30 | .102 | .102 | .078 | .086 |
| 60 | .173 | .190 | .167 | .142 |
| 90 | .275 | .277 | .255 | |
| 120 | .362 | .372 | .352 | .292 |
| 150 | .444 | .456 | .462 | .456 |

### Reproducibility of Results

On the basis of a 3-month period, and using different batches of reagents, series of standard solutions were analyzed and a confidence limit of ± 2.8 mg./100 ml. was calculated (Table 3).

### Stability of Reagents

Standard urea solutions from 30 to 150 mg./100 ml. were analyzed with the same reagents for a period of 20 weeks (Table 4). No variations have been observed regarding the stability of reagents kept in brown bottles at room temperature. The addition of phosphoric acid to DAM-TSC mixture produces a loss of sensitivity to the reaction after 3 hr. After 3 days, no reaction at all was possible. We recommend addition of acid to the color-producing reagents just before use.

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# BOOK REVIEWS

**Methods of Biochemical Analysis.** D. GLICK (Ed.) Interscience Publishers, Inc., New York, 1961, vol. 9, ix + 452 pp. $14.50.

Volume 9 adds a new member of equal quality and usefulness to the *Methods of Biochemical Analysis* series. While none of the topics reviewed could be directly applied to the routine of clinical chemistry, several carry seeds which may develop with unexpected velocity into procedures for the clinical laboratory, considering the signal acceleration of evolution from fundamentals to specific technics due to automation and to modern instrumentation in general. The article by Carstensen on countercurrent distribution for analysis of adrenal steroids in blood is a case in point. The short reviews of electrophoresis in agarose and on reflection spectroscopy open new aspects in methodology. The papers on assay of deoxyribonuclease and ribonuclease supplement each other in their timeliness. More than 40% of the text is taken up by a discussion of nuclear magnetic resonance and electron spin resonance by O. and C. D. Jardetzky. One may think that a monographic treatise of nearly 200 pages unduly exceeds the frame of this or similar series, but the Editor is fully justified in according this extra space to a method of such momentous impact on organic chemistry and in offering a very readable digest of these technics to biochemists.

*Mt. Sinai Hospital, New York, N. Y.* HARRY SOBOTKA

# LETTERS TO THE EDITOR

*To the Editor:*

The *p*H of a buffer should be measured and specified at the exact temperature of use. The *p*H optima of enzyme systems should be measured in and specified for the final reaction mixture at the reaction temperature. Room temperature is never an adequate specification, since it may vary from 15°C to 35°C.

The following examples from two papers on serum lipase illustrate the problems created by inadequate specification of temperature. These examples are not meant to detract from the excellence of the work reported. Failure to report *p*H values of buffers and reaction mixtures at the temperature of use is so widespread that almost any other papers would have served as well.

Tietz, Borden, and Stepleton (*Am. J. Clin. Path.* **31**:148, 1959) recommended a 0.2M tris (hydroxymethyl) aminomethane buffer of *p*H 8.0 for serum lipase determinations. They did not specify the temperature at which *p*H 8.0 was determined. If, for example, the buffer was *p*H 8.0 at 25°, then at 37° it would have been *p*H 7.71. This difference is important because the *p*H maximum of serum lipase is sharply defined. The authors showed that a change in buffer *p*H of 0.3 units caused a twofold change in hydrolysis rate.

Tietz, Borden, and Stepleton realized that the actual *p*H of the test mixture was different from the *p*H of the buffer used, but they failed to specify the temperature at which the *p*H of the test mixture was measured. The temperature coefficient of serum is large, increasing about 0.01 *p*H units for each degree Celsius decrease. This serum *p*H change would be in addition to the TRIS *p*H change with temperature. The authors showed that a 0.1 *p*H change in the test mixture caused a twofold change in hydrolysis rate. They specified *p*H 7.82 as the optimum for the final test mixture, but temperature of measurement was not specified. Therefore, it is impossible to determine whether this was the optimum *p*H at 37°. From a description of the equipment used it would appear that *p*H was measured at "room temperature."

MacDonald and Le Fave (*Clin. Chem.* **8**:509, 1962) reported on an improved and more rapid lipase method; and they too recommended a TRIS buffer of *p*H 8.0. However, these authors also failed to specify the temperature of measurement. MacDonald and LeFave recommended checking the *p*H of the TRIS buffer with a *p*H meter and suggested adjusting the *p*H if it was found to differ from 8.0. This would only compound the problem. If *p*H 8.0 is specified for 37°, and room temperature is 22°, then adjustment of the *p*H to 8.0 units at room temperature would cause the *p*H to be 7.62 at 37°. If, on the other hand, *p*H 8.0 is specified for an average room temperature, then the specified *p*H optimum would be erroneous at 37°.

The proper solution is for all chemists to measure and report the *p*H of buffers

at the exact temperature of the final reaction mixture. In addition, all chemists should measure and report the *p*H of the final reaction mixture itself at the reaction temperature.

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*Englewood, N. J.*

*Dear Sir:*

Highly objectionable and erroneous implications were made in the Scientific Note entitled "Use of —log(—log T) or —log A Tables in Iodide Analysis by Catalytic Ceric-Ion-Reduction Method," which appeared in the April 1962 issue of CLINICAL CHEMISTRY (Vol. 8, p. 194). After a rather torturous and ambiguous treatment of first-order kinetics, the author derived a straight-line relationship between —log. A (absorbancy) of ceric ion and concentration of iodide catalyst, and stated that *his* theory had been confirmed by Dr. Boutwell. Furthermore, he stressed the superiority for quantitative PBI determination of the —log. A relationship to iodide over the straight-line relationship between the transmittancy and concentration of iodide catalyst. Finally, the author presented a table for conversion of transmittancy values to values proportional to —log.(—log. T), thus accomplishing the conversion commonly accomplished by a graphic method, i.e., a plot of A on semilogarithmic paper against either time at constant iodide concentration or iodide concentration at constant time. The objectionable and erroneous implications are as follows:

1. The method of presentation suggests that this was a newly discovered relationship, but as a matter of fact the straight-line relationship between —log. A and iodide concentration has been in general use for quantitative PBI determination by graphic methods for over 11 years.

2. The derivation presented is invalid. The author assumes that the ceric ion in solution obeys Beer's law, but it is a well-documented and readily confirmed fact that a plot of A against ceric-salt concentration gives a curve of sigmoid form.

3. The assumed superiority of the —log. A vs. iodide over T vs. iodide is not valid in the absence of experimental proof since both empirical straight-line relationships hold over a limited range of ceric-ion concentrations.

4. More than half of the table presented is not only useless but misleading because it covers regions in which a linear relationship does not exist.

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*To the Editor:*

The writer appreciates the opportunity (*1*) to expand his note on use of the $-\log_{10}(-\log_{10}T)$ relationship (*2*) and to give experimental data on the breadth of its validity.

1. *Test of the validity of the Beer-Lambert relationship with respect to low concentrations of ceric ion.* In this work the ceric salt used was furnished by the G. Frederick Smith Chemical Co. as ceric sulfate, $Ce(HSO_4)_4$. The stock solution was prepared on the assumption that the salt was pure. Inasmuch as dilutions were made from the same stock solution, an error in its strength would not affect conclusions.

When organic matter is ashed in the presence of sodium carbonate, which is then dissolved in HCl, large amounts of chloride are present along with iodide. Chloride was therefore included in the dilutions of ceric sulfate used in testing the absorbance-concentration linearity, each solution being 0.3 *M* in NaCl and 1 *N* in $H_2SO_4$. The absorbances (relative to water) of these solutions were determined in an Evelyn-type photometer, using matched 15-mm. Pyrex test tubes, and a Corning light filter combination transmitting 17% of light at 430 mμ, with a half-maximum band width of 30 mμ. Absorbancies (relative to water) of the same solutions were also determined in a Beckman DU Spectrophotometer, using 10-mm. "square" Vycor cuvets, at a wave length of 430 mμ, with a slit width of about 0.034 mm. Results plotted in Fig. 1 show a mediocre conformity to the Beer-Lambert relationship in the Evelyn-type instrument, confirming Dr. Fales' contention (*1*) for this instrument. That the failure to ob-

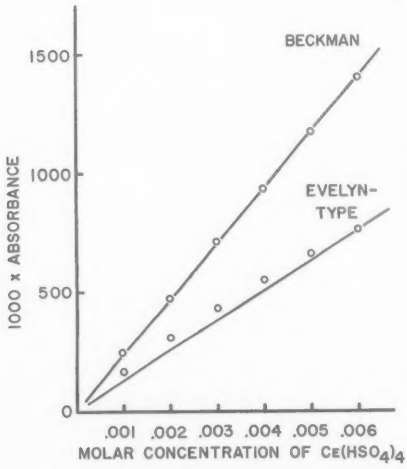


**Fig. 1.** Conformity of ceric salt solution to Beer-Lambert relationship, using a glass-filter photometer and a prism spectrophotometer.

tain a straight line is largely due to an instrumental deficiency is, however, indicated by the much better conformity obtained with the Beckman instrument. The poorer results from the instrument employing a glass filter are doubtless related to wider transmittance on the steep slope of the ceric ion transmittance curve producing a stray-light effect. This effect is minimized by use of an instrument employing a narrow band of light frequencies. The Beer-Lambert relationship then becomes satisfactory.

2. *Test of the range of transmittances over which* $-log_{10}(-log_{10}T)$ (LLT) *gives a straight line relationship.* In view of the results obtained above, subsequent transmittances were determined in a Beckman DU Spectrophotometer under the conditions noted above. Room temperature at the time of the experiment was 27.2° ± 0.2°.

Six flasks were prepared containing a mixture of aqueous solutions of KI, $As_2O_3$, NaCl, and sulfuric acid, such that when 2.00 ml. of $Ce(HSO_4)_4$ solution was added with mixing on a timed schedule, each flask then contained 10 ml. of a solution 0.01 *M* in $Ce^{++++}$ salt, 0.01 *M* in $As^{+++}$, 0.2 *M* in NaCl, and 1.0 *N* in $H_2SO_4$. The iodide concentrations varied from zero (blank) to 0.010 μg. of $I^-$ per milliliter. Some of each solution was transferred to respective Vycor cuvets, and transmittances were read at 10-min. intervals following addition of the ceric salt, for 60 min. Results of this experiment are shown in Fig. 2, 3, and 4, each of which presents the same set of data in a different way.

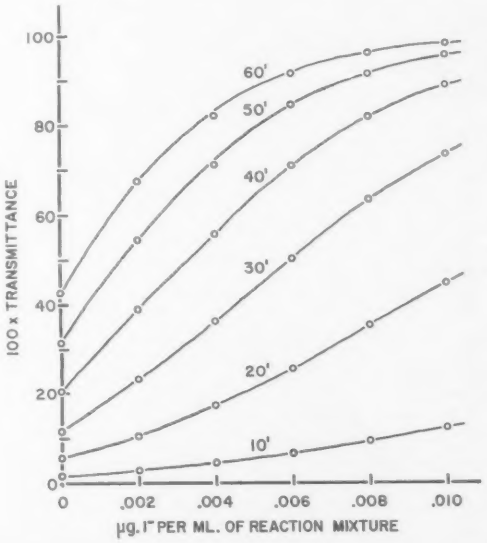


**Fig. 2.** Change of transmittance with iodide concentration after various time intervals. Data plotted as 100 × transmittance at various time intervals versus μg. $I^-$ per milliliter of reaction mixture in the cuvet. (100 × transmittance = %T.)

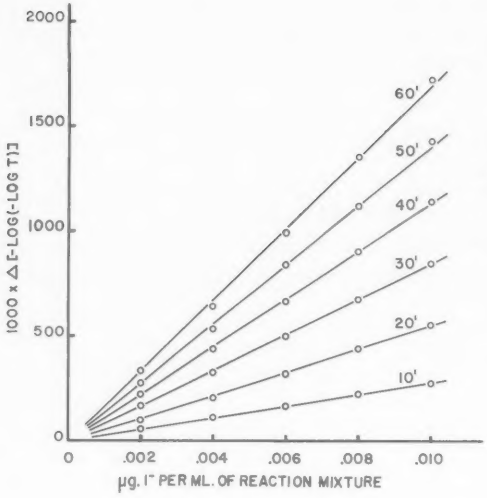


**Fig. 3.** Change in —log(—log T) with iodide concentration for fixed time interval. Increase in —log(—log T) values for each cuvet above those of the blank cuvet read at the same time intervals.

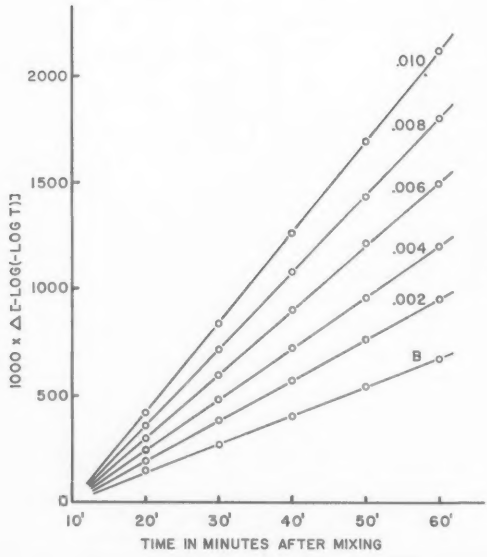


**Fig. 4.** Change in —log(—log T) with time at fixed iodide concentration. Increase in —log (—log T) values for each cuvet, read at indicated time intervals, above —log(—log T) value of same cuvet read 10 min. after addition of ceric sulfate.

Figure 2 shows transmittances, varying from 1.8 to 98.4%, plotted against iodide concentrations. As Doctor Fales notes, in this manner one obtains only an approximation to a straight-line relationship, and this only over the central group of points.

Figure 3 shows the increase in the values of *LLT* of each sample above the values of the blank at the same time intervals, plotted against iodide concentrations ($LLT^t_x - LLT^t_b$), each line representing a single value of $t$. Figure 4 shows the increase in *LLT* for each cuvet above its 10-min. value, plotted against time ($LLT^t_x - LLT^{10'}_x$), each line representing a single value of $x$. Since the published table (*2*) of *LLT* values extends only as low as 10% transmittance, it was necessary to look up the logarithms of the absorbancies for a number of the values. (Values of the *LLT* function become negative below $T = 0.1$, and have to be added or subtracted algebraically.) In view of the results obtainable, it may be that the published table is not as extensive as it should be.

Figures 3 and 4 indicate that, whether iodide concentration or elapsed time is chosen as the parameter, a straight-line relationship can be secured over a wide range of transmittances if proper conditions are met. In practical use this theoretical range is subject to analytical variations and methodological limitations. Such possible variations and limitations may of course limit the accuracy attainable at extreme values, but experimental evidence here presented speaks strongly for the validity of the mathematical relationship derived in the published note (*2*). Some analysts may prefer to use this mathematical relationship in their calculations, rather than to employ semilogarithmic paper, use of which rests on identical theoretical relationships.

*Temple University School of Medicine* ROBERT HOUSTON HAMILTON
*Philadelphia 40, Pa.*

## References

1. Fales, F. W. Letter to the Editor, above.
2. Hamilton, R. H., *Clin. Chem.* **8**, 194 (1962).

the Clinical Chemist

# THE AMERICAN ASSOCIATION OF CLINICAL CHEMISTS
# and
# THE CANADIAN SOCIETY FOR CLINICAL CHEMISTRY

## Fifth International Congress

### Statler-Hilton Hotel, Detroit, Mich.
### Aug. 19–23, 1963

The Congress is organized by the American Association of Clinical Chemists and the Canadian Society for Clinical Chemistry under the authority of the International Federation of Clinical Chemistry and the Commission on Clinical Chemistry of the International Union of Pure and Applied Chemistry.

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## Scientific Program

The four morning programs will consist of symposia with invited speakers. The provisional program follows.

*Metabolic Diseases*
CHAIRMAN: S. H. JACKSON (Toronto)
SPEAKERS: G. LATHE (Leeds)
A. SASS-KORTSAK (Toronto)
O. WIELAND (Munich)

*Lipid Metabolism*
CHAIRMAN: D. B. TONKS (Toronto)
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*Enzymes in Clinical Chemistry*
CHAIRMAN: J. G. REINHOLD (Philadelphia)
SPEAKERS: Y. HATEFI (Shiraz)
K. S. HENLEY (Ann Arbor)
B. HESS (Heidelberg)

*Physical Methods in Clinical Chemistry*
CHAIRMAN: B. ZAK (Detroit)
SPEAKERS: O. MULLER (New York)
S. NATELSON (New York)
A. ZLATKIS (Houston)

The four afternoon sessions will be composed of communications submitted by the members of the Congress. Each member of the Congress is entitled to submit an abstract of one paper for presentation. It is anticipated that at least three sections will meet simultaneously. Each paper and the ensuing discussion will be limited to 15 min. In order to prevent overcrowding of the program the committee may assign certain papers to be "read by title" only; however, all abstracts will be published in CLINICAL CHEMISTRY. Papers and abstracts may be presented in English, French, or German, but English is most likely to be generally understood. There will be no simultaneous translation service.

## Submission of Abstracts

Three copies of an abstract of not more than 250 words must be in the hands of the Program Chairman, DR. SANFORD H. JACKSON, Hospital for Sick Children, Toronto 2, Ontario, Canada, by Mar. 1, 1963. Illustrations, tables, and references cannot be included in the abstracts. Membership in the Congress must be established by payment of the registration fee before the communication will be considered for inclusion in the program.

## Projection Facilities

Slide projectors will be available for $5 \times 5$-cm. ($2 \times 2$-in.) slides *only*.

## Technical Exhibition

A large exhibition of laboratory apparatus, chemical reagents, technical books, etc., will be open daily in Hall D of Cobo Hall. Further information concerning the exhibition can be obtained from the Exhibition Manager, EMANUEL EPSTEIN, Ph.D., Depart-

ment of Clinical Pathology, St. Joseph Mercy Hospital, Pontiac, Michigan, U.S.A.

No facilities will be available for demonstrations by the members of the Congress.

## Tentative Program

**Sunday, August 18**

1 P.M.–9 P.M.: Registration, Statler-Hilton Hotel
7 P.M.–10 P.M.: Social hour

**Monday, August 19**

8 A.M.: Registration; Cobo Hall
9 A.M.: Opening ceremony, Symposium
2 P.M.: Sectional meetings
8 P.M.: Reception
NOON–4:30 P.M.: Technical exhibit

**Tuesday, August 20**

9 A.M.: Symposium
2 P.M.: Sectional meetings
8 P.M.: Discussion of standardization and controls
9 A.M.–4:30 P.M.: Technical exhibit

**Wednesday, August 21**

Free day for sightseeing excursions

**Thursday, August 22**

9 A.M.: Symposium
2 P.M.: Sectional meetings
7 P.M.: Congress diner
9 A.M.–4:30 P.M.: Technical exhibit

**Friday, August 23**

9 A.M.: Symposium
2 P.M.: Sectional meetings
5 P.M.: Closing ceremony
9 A.M.–NOON: Technical exhibit

## General Information

### Location

All scientific sessions of the Congress and the technical exhibition will be held in Cobo Hall. The reception and dinner of the Congress will be held in the Statler-Hilton Hotel.

### Registration

Membership in the Congress is open to all persons interested in clinical chemistry. The registration fee of $15 (U.S.) includes admission to all scientific meetings, technical exhibition, reception, etc., but not to the Congress Dinner or excursions.

Associate membership is open to those accompanying full members but not attending the scientific meetings. The registration fee of $5 (U.S.) includes admission to the opening and closing ceremonies, reception, and certain events arranged by the Ladies' Committee. It does not include the cost of the Congress Dinner or the special excursions.

Advance registration will be possible for applications received by the Secretary before Aug. 1, 1963.

Checks, bank drafts, or international money orders should be made payable to: Fifth International Congress on Clinical Chemistry.

### Passports and Visas

All travelers from outside North America will be required to carry valid passports and the necessary visas. Those planning to attend the Stratford Festival on the special excursion are reminded that a re-entry

visa will be required for return to Detroit.

### Hotel Accommodations

All arrangements for reservations in hotels or motels are to be made directly. The principal hotels near Cobo Hall and their rates are listed below.

| *Hotel* | *Single* | *Double or Twin* |
|---|---|---|
| Statler-Hilton | $7.50–14.00 | $13.00–25.00 |
| Detroit Leland | $7.00–13.50 | $10.00–19.00 |
| Henrose | $6.50–10.50 | $ 9.00–16.00 |
| Pick-Fort Shelby | $6.00–13.00 | $10.00–17.00 |
| Sheraton Cadillac | $7.00–18.00 | $13.00–23.00 |

For those traveling by automobile and wishing to stay in motels, information can be obtained from travel agents, automobile clubs, or the Detroit Convention and Tourist Bureau, Detroit 26, Mich. There are many motels located so that travel to Cobo Hall is convenient. All-day parking is available at Cobo Hall.

### Dress

Academic dress or evening dress will not be required at any function.

### Other Meetings

A symposium entitled "Proteins, Genes and Evolution" will be presented in Toronto on Saturday morning, Aug. 17, 1963, by speakers from the hospitals, the University of Toronto, and the Connaught Laboratories. For further information write to DR. C. J. PORTER, Department of Biochemistry, Toronto General Hospital, Toronto 2, Ontario, Canada.

Following the Congress, the 145th national meeting of the American Chemical Society will be held in New York between September 8 and 13. Information on the program will be found in *Chemical and Engineering News*.

### Special Excursion

A special excursion to Stratford, Ontario, to attend the matinee performance of the Shakespeare Festival Theatre on Wednesday, August 21, will be organized. Busses will leave the Statler-Hilton Hotel at 7:30 A.M. and return at approximately 10:30 P.M. The cost per person, including transportation, lunch, admission, and dinner, will be $15 (U.S.). A maximum of 390 persons can be accommodated for this trip. Further information or reservations can be obtained from DR. SANFORD H. JACKSON, Hospital for Sick Children, Toronto 2, Ontario, Canada.

### Further Information

Additional copies of this announcement, application forms, or further information concerning the Congress can be obtained from the Congress Secretary, DR. DONALD G. REMP, Fifth International Congress on Clinical Chemistry, Henry Ford Hospital, Detroit 2, Mich., U.S.A.

## News Section

### Section News

#### Philadelphia Section

The topic of the 82nd scientific program, held Nov. 27, 1962, was "Assessment of the Plasma Hydrocortisone Level in Man," and was presented by DR. HAROLD PERSKY, formerly at the University of Indiana, now at the Albert Einstein Medical Center.

After a brief historical resume of methodologies, some problems were pointed out (such as the interference from chlorpromazine), and then material related to the clinical aspects of the problem was discussed. It was noted that diurnal variation existed which was not changed in night workers. A limitation in interpretation was suggested by the fact that increased turnover might exist without a concomitantly large rise in the plasma concentration. The effects of some disease situations as well as surgical and psychological stress were considered.

The subsequent ChemiCliniC program was centered on problems of instrument control and the possibility of defining in some physical terms the response of spectrophotometers, hence, comparison of a number of like instruments was made, with similarities and differences noted. DR. CARL ALPER, of Hahnemann Medical College and Hospital, was the speaker.

# Standard of Purity for Cholesterol

*Nathan Radin and Adalbert L. Gramza*

**Three recrystallization technics were used to fractionate five commercial cholesterol products and one artificial mixture. One technic involved the recrystallization of cholesterol from absolute ethyl alcohol, the second technic involved the recrystallization of cholesterol from glacial acetic acid, and the third technic used the dibromide derivative method. The molar absorptivities for the various crystal batches were in the range of 1610 to 1750 L. mole$^{-1}$ cm.$^{-1}$ at 620 m$\mu$ with a modified Liebermann-Burchard procedure. The molar absorptivities were in the range of 9,800 to 11,500 L. mole$^{-1}$ cm.$^{-1}$ at 560 m$\mu$ with a modified sulfuric acid-iron method. The original products and the ethyl alcohol recrystallized products showed molar absorptivities at the lower limits of the ranges, while the glacial acetic acid and dibromide derivative recrystallized cholesterol showed molar absorptivities at the higher limits of the ranges. Absorption peaks at 235 m$\mu$ for methyl alcohol solutions of the cholesterol preparations were used to estimate cholesterol impurities on the basis of 7-keto cholesterol acetate absorption values. The decrease of absorption in the ultraviolet spectral region established that impurities were removed from the cholesterol preparations studied.**

THE SERUM CHOLESTEROL determination presents many problems as shown by discussion and the many papers covering the subject. The subject of cholesterol as a primary standard is usually not discussed in cholesterol methodology articles. A reliable criterion for establishing the purity of cholesterol has not been established.

The investigation reported in this paper is concerned with the evaluation of the purity of a number of commercial cholesterol preparations, preparation of pure cholesterol, and a study of the properties of the purified cholesterols.

From the Division of Biochemistry, Rochester General Hospital, Rochester 8, N. Y.

Presented at the Fourteenth Annual Meeting of the American Association of Clinical Chemists, Aug. 28, 1962, Santa Monica, Calif.

The authors wish to thank Galla Radin for her aid in the statistical analyses of our data.

Received for publication Oct. 29, 1962.

## Experimental

### Recrystallization and Characterization of Commercial Cholesterol Preparations

Commercial cholesterol is usually prepared by solvent extraction of spinal cords and brains of cattle (*1*). Cholesterol is then crystallized and recrystallized directly or after the esterified cholesterol has been saponified with alkali. Information from various commercial sources for laboratory quantities of cholesterol shows that the U.S.P. specifications (*2*) are now the accepted standard for cholesterol purity.

#### Cholesterol Recrystallization

The meaning of *pure* leads to various definitions, starting with: a pure compound is one in which all molecules are identical. In this investigation the definition of purity used is in terms of operations to be performed. A system of molecules is a pure compound if an exhaustive series of fractionations fails to produce fractions with different properties. What one calls a pure compound thus changes as new methods become available for separating material into fractions, or for more accurately measuring the properties of the fractions (*3*). The guiding principle in the present study was to prepare and evaluate cholesterol as a primary standard using the equipment available within our laboratory.

As usually prepared, cholesterol is contaminated by cholestanol (dihydrocholesterol or cholestan-3 $\beta$-ol), cholest-7-en-3 $\beta$-ol (lathosterol or $\Delta^7$-cholestenol), and traces of 7-dehydrocholesterol (cholesta-5, 7-dien-3 $\beta$-ol) (*1*).

Three recrystallization methods were chosen for this study. One involved the recrystallization of cholesterol from ethanol, one involved the recrystallization of cholesterol from acetic acid (*4*), and one involved the treatment of cholesterol in ether solution with bromine, which precipitates the insoluble 5, 6 $\beta$-dibromide derivative, which is then regenerated with zinc and acetic acid (*5*). According to Fieser (*6*) crystallization of cholesterol from ethanol does not remove the cholest-7-en-3 $\beta$-ol and cholestanol present, but purification through the dibromide eliminates these companions as well as two products of air oxidation, 7-ketocholesterol and cholestane-3 $\beta$, 25-diol.

#### Cholesterol Recrystallization from Ethanol

Cholesterol was added in the proportion of 1 gm. per 5 ml. to absolute ethanol (Publicker). A beaker containing the mixture was heated

on a hot plate, with mixing by swirling until the cholesterol was dissolved. The beaker was then removed from the hot plate, covered with a watch glass, and was allowed to cool at room temperature. The cholesterol which precipitated was separated by filtration through Whatman No. 1 filter paper in a Buchner funnel. The damp crystals were washed with a small volume of diethyl ether, dried overnight, then dried in an oven at 90° for 2 hr., and finally stored over phosphorus pentoxide in a vacuum desiccator.

#### Cholesterol Recrystallization from Acetic Acid

Glacial acetic acid (Baker's, ACS) was heated to the boiling point in an Erlenmeyer flask and then poured into a beaker which contained cholesterol. Eight milliliters of acetic acid was used for each gram of cholesterol. The mixture was stirred vigorously with a wooden paddle until the solid material was all dissolved. The beaker was then plunged into an ice bath, the contents being stirred vigorously. When room temperature was attained the complex was collected on Whatman No. 1 filter paper in a Buchner funnel. After a washing with acetic acid and methanol, the crystals were air dried overnight, and then dried in an oven at 90° for 2 hr. The cholesterol was then stored in a vacuum desiccator over phosphorus pentoxide.

#### Recrystallization of Cholesterol with Dibromide Method

Fifty grams of cholesterol was dissolved in 350 ml. of ethyl ether by gentle warming on a steam bath. After cooling the resultant solution to room temperature, a solution containing 22.7 gm. bromine, 1.67 gm. anhydrous sodium acetate, and 200 ml. of glacial acetic acid was added. A white paste resulted. The mixture was stirred about 10 min., during which time it was cooled to 20°. The material was transferred to a Buchner funnel containing Whatman No. 1 filter paper and was washed with acetic acid until the filtrate was colorless. The white material was then suspended in 750 ml. of diethyl ether and 10 ml. of glacial acetic acid. Ten grams of zinc dust was slowly added and a reaction was evidenced by bubbles of hydrogen. A white precipitate formed which consisted of zinc salts, and this was dissolved by the addition of 10 ml. of water. The ether solution was then decanted from the water layer containing the excess solid zinc. By use of separatory funnels the ether solution was washed twice with 150-ml. portions of acid solution (6 ml. of concentrated hydrochloric acid for each 100 ml. of water) and then washed with 10% (w/v) sodium hydroxide solution

to remove the acetic acid. Five hundred milliliters of methanol was then added to the ether solution and the resulting solution was evaporated slowly on the steam bath to the point where most of the ether was removed and purified cholesterol began to crystallize. Crystallization proceeded at room temperature. The product was collected, air dried overnight, dried in an oven at 90° for 2 hr. and then stored in a vacuum desiccator over phosphorus pentoxide.

### Characterization of Recrystallized Cholesterol

The commercial cholesterol preparations chosen for this study were from Armour Pharmaceutical Company, Pfanstiehl Laboratories, Inc., Steraloids, Inc., General Biochemicals, Inc., and K & K Laboratories, Inc. The commercial cholesterol preparations were all white, powdery substances except for the K & K preparation, which was yellow. A mixture of cholesterol was made by combining and mixing an old yellow Eastman preparation with equivalent amounts of material from the other companies and heating at 100° for 6 hr. After heating, the cholesterol pool was dark yellow.

Anhydrous cholesterol is obtained by crystallization from dry solvents and forms triclinic needles. Cholesterol separates as the monohydrate, rhombic-shaped, triclinic plates from moist solvents (*1*). The ethanol-recrystallized cholesterol formed a white, fluffy crystalline material; the acetic acid-recrystallized cholesterol formed a white, packed material; and the cholesterol from the dibromide recrystallization method came out as a lustrous white powder. The yellow color of the K & K product and the mixture was not completely removed with recrystallization from ethyl alcohol or acetic acid.

The cholesterol monohydrate loses water at 70–80° (*1*). Thus, in order to ensure anhydrous cholesterol after air drying, the recrystallized materials were heated in an oven at 90° for 2 hr. The cholesterol preparations treated this way showed no change from the original appearance. However, it would be safer to use a vacuum oven for this operation.

The weight changes due to the loss of moisture after heating and drying known amounts of the commercial cholesterol preparations were shown to be negligible.

The melting point of all the preparations are shown in Table 1. The method for obtaining the melting points in evacuated capillaries was described by Fieser (*7*). Fieser has observed that melting points taken in open capillaries are often several degrees lower than those

**Table 1.** Melting Points of Commercial and Recrystallized Cholesterol

| *Commercial product* | *Original* (°C.) | *EtOH 1X* (°C.) | *EtOH 2X* (°C.) | *HAc 1X* (°C.) | *HAc 2X* (°C.) | *DiBr 1X* (°C.) | *DiBr 2X* (°C.) |
|---|---|---|---|---|---|---|---|
| Armour Lot V 31105 | 148.7–150.5 | 148.8–151.8 | 149.7–151.8 | 149.4–151.3 | 150.3–150.9 | 149.6–151.3 | 149.9–150.9 |
| Pfanstiehl Lot 4907 | 148.2–150.0 | 147.9–150.6 | 147.7–150.3 | 149.5–151.7 | 149.4–151.3 | 148.3–151.0 | 149.0–151.8 |
| Steraloid Batch 62453 | 148.2–150.8 | 148.7–151.6 | 149.0–151.8 | 149.2–150.9 | 149.6–151.7 | 148.8–151.2 | 148.8–151.2 |
| K & K Lot 5550 L | 146.5–150.2 | 147.6–151.9 | — | 147.8–151.2 | — | 148.2–151.8 | — |
| General Biochemicals 6071 I | 142.6–148.2 | 147.9–151.1 | — | 147.8–151.5 | — | 147.6–150.8 | — |
| Mixture | 137.5–144.1 | 146.9–149.2 | — | 145.9–149.5 | — | 148.6–151.9 | — |

taken in evacuated capillaries. Fieser has shown that the melting point for cholesterol, purified by recrystallization from acetic acid is 14.95–150.0° (*4*).

## Determination of Absorptivities of Commercial and Recrystallized Cholesterol

The spectrophotometric technic for characterizing the cholesterol products under study was chosen as the equipment is generally available in the clinical chemistry laboratory. The extinction coefficient for cholesterol in ethanol in the ultraviolet region of the spectrum has been reported (*8*). As the maximum of the absorption peak for cholesterol is reported at 203 mμ any measurements made with the Beckman Spectrophotometer, Model DU, in the spectral region with the wavelength less than 210 mμ would be open to question. The molar absorptivities were determined for two commonly used color reactions, the Liebermann-Burchard reaction and the sulfuric acid-iron reaction. The Beckman Spectrophotometer, Model DU, was used for measuring absorbancies, and the Model DB was used for recording wavelength-transmittance curves. The cell compartments were kept at 25° with water circulating from a thermostat. Ten-millimeter light-path-matched cells (Quaracell, OS and QS) were used for the spectrophotometric studies.

### Liebermann-Burchard Reaction

A modified Liebermann-Burchard procedure was adapted from the Carr and Drekter (*9*) cholesterol technic. The procedure used to measure the absorbance values and to calculate the molar absorptivities was as follows:

1. About 0.1000-gm. portions of the cholesterol samples were weighed, using weighing boats and were then transferred to 100-ml. volumetric flasks, using glacial acetic acid (Baker, ACS) as the wash solvent. The flasks were kept at room temperature overnight to permit complete solution of the cholesterol preparations. The solutions were then diluted to mark with glacial acetic acid and mixed well.

2. Five-milliliter aliquots of each stock solution were pipetted into 50-ml. volumetric flasks, and 5 ml. of acetic acid was pipetted into another 50-ml. volumetric flask for the reagent blank.

3. Twenty-milliliter aliquots of acetic anhydride (Baker) were pipetted into each volumetric flask.

4. A 20% sulfuric acid-glacial acetic acid solution (V/V) was

poured up to about the neck in the first flask as a stop watch was started. After swirling to mix, the flask was placed in a water bath at 25°. The same procedure was followed with other flasks at exactly 1-min. intervals. Each solution was diluted to mark with the acid mixture, mixed, and returned to the water bath at exactly 10 min. after the initial operation.

5. The absorbance of each solution was measured at 620 m$\mu$ exactly 20 min. after the initial operation.

6. The molar absorptivity was calculated for each solution using the Bouguer-Beer Law, $A = abc$, where $A$ is absorbance, $a$, molar absorptivity, $b$, light path in centimeters, and $c$, concentration of the final solution in moles per liter.

For each commercial or recrystallized product two stock cholesterol solutions were prepared at different times. Duplicate absorbance measurements of the color reaction described were made for each stock solution 3 times. Thus, for each cholesterol preparation there was a total of 12 absorbance measurements, and 12 molar absorptivities were calculated.

The wavelength-transmittance curve for this reaction (Fig. 1) shows a maximum absorption peak between 620 and 630 m$\mu$. The shapes of the curves were essentially the same whether the cholesterol was a commercial preparation or a recrystallized product. Running the curves sequentially showed that there was about a 1% transmittance change for every 11 min. By maintaining exact intervals during color development and absorbance readings equal periods for color development was assured. The relation between absorbance and cholesterol was shown to be linear at 620 m$\mu$. Table 2 shows the mean

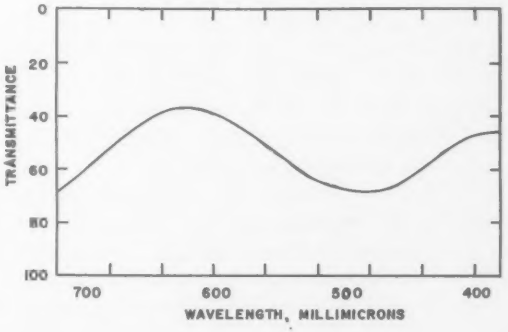


**Fig. 1.** Wavelength-transmittance curve for the Liebermann-Burchard cholesterol procedure.

**Table 2.** Molar Absorptivities for the Liebermann-Burchard Cholesterol Procedure

| *Commercial product* | *Original* ($a^*$) | *EtOH 1X* ($a^*$) | *EtOH 2X* ($a^*$) | *HAc 1X* ($a^*$) | *HAc 2X* ($a^*$) | *DiBr 1X* ($a^*$) | *DiBr 2X* ($a^*$) |
|---|---|---|---|---|---|---|---|
| Armour Lot V 31105 | 1650 | 1670 | 1660 | 1670 | 1690 | 1700 | 1690 |
| Pfanstiehl Lot 4907 | 1660 | 1710 | 1710 | 1690 | 1700 | 1700 | 1710 |
| Steraloid Batch 4907 | 1720 | 1730 | 1730 | 1720 | 1740 | 1750 | 1740 |
| K & K Lot 5550 L | 1700 | 1740 | — | 1740 | — | 1740 | — |
| General Biochemicals 6071 I | 1650 | 1720 | — | 1740 | — | 1750 | — |
| Mixture | 1610 | 1690 | — | 1730 | — | 1740 | — |

*Molar absorptivity: liters cm. $^{-1}$ moles $^{-1}$. Each value is the mean of 12 single measurements.

molar absorptivity values for commercial and recrystallized cholesterol products.

### Sulfuric Acid-Iron Reaction

The procedure shown below, for the sulfuric acid-iron reaction, was adapted from the Rosenthal, Pfluke, and Buscaglia (*10*) cholesterol technic.

1. Three-milliliter aliquots of the cholesterol stock solutions prepared for the Liebermann-Burchard reaction measurements were diluted to mark in 100-ml. volumetric flasks with glacial acetic acid.

2. A stock reagent was prepared by dissolving and diluting 5 gm. of ferric chloride ($FeCl_3 \cdot 6\ H_2O$, Baker, ACS) with concentrated ortho-phosphoric acid (85%, Baker, ACS) to mark in a 50-ml. volumetric flask. Five milliliters of the stock reagent and 15 ml. of concentrated phosphoric acid were diluted to mark with concentrated sulfuric acid in a 250-ml. volumetric flask for the coloring reagent. This dilute coloring reagent was freshly prepared every day that measurements were made.

3. Five-milliliter aliquots of each diluted cholesterol solution (Step 1) were transferred into 50-ml. Erlenmeyer flasks. Five milliliters of glacial acetic acid was transferred into a flask for the blank. The flasks were then placed in a water bath at 25°. Four milliliter aliquots of the dilute coloring reagent was transferred into each flask at minute intervals and mixed by swirling.

4. The absorbancies for each solution were measured at 560 m$\mu$ 30 min. from the initial addition of the dilute coloring reagent.

5. The molar absorptivities were calculated using the Bouguer-Beer Law as described for the Liebermann-Burchard reaction. However, in this case the concentration value was based on a 9-ml. final

volume which was not corrected for any volume change that took place when the glacial acetic acid and sulfuric acid solutions were mixed.

A typical wavelength-transmittance curve for the cholesterol sulfuric acid-iron reaction is shown in Fig. 2. There is a maximum absorption peak between 540 and 560 m$\mu$. The spectral curves recorded for this study reproduce the spectral curves shown by Rosenthal and Jud (*11*). The shapes of the curves were essentially the same whether the cholesterol examined was a commercial or a recrystallized product. Running the curves sequentially showed that there was a 1% transmittance change every 11 min. Thus, for this reaction, as with the Liebermann-Burchard reaction, equal intervals of color development and absorbance measurements were maintained. The relation between absorbance and cholesterol concentration at 560 m$\mu$ was shown to be linear. Table 3 shows the mean molar absorptivity values for the commercial and recrystallized cholesterol products. Each mean molar absorptivity value represents 12 values obtained as described for the Liebermann-Burchard procedure.

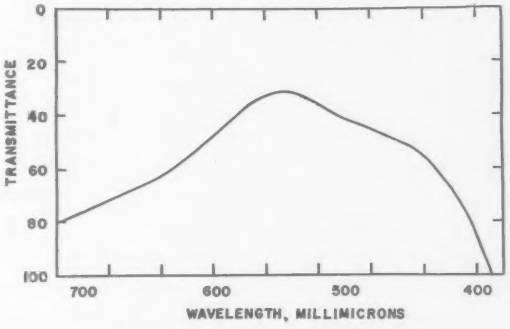


**Fig. 2.** Wavelength-transmittance curve for the sulfuric acid-iron cholesterol procedure.

**Table 3.** Molar Absorptivities for the Sulfuric-Iron Cholesterol Procedure

| *Commercial product* | *Original* ($a^*$) | *EtOH 1X* ($a^*$) | *EtOH 2X* ($a^*$) | *HAc 1X* ($a^*$) | *HAc 2X* ($a^*$) | *DiBr 1X* ($a^*$) | *DiBr 2X* ($a^*$) |
|---|---|---|---|---|---|---|---|
| Armour Lot V 31105 | 11100 | 11100 | 11200 | 11300 | 11300 | 11300 | 11300 |
| Pfanstiehl Lot 4907 | 10800 | 11200 | 11300 | 11200 | 11300 | 11300 | 11300 |
| Steraloid Batch 4907 | 11300 | 11400 | 11400 | 11500 | 11500 | 11400 | 11500 |
| K & K Lot 5550 L | 11000 | 11200 | — | 11400 | — | 11500 | — |
| General Biochemicals 6071 I | 10700 | 11100 | — | 11400 | — | 11400 | — |
| Mixture | 9800 | 11000 | — | 11200 | — | 11300 | — |

*Molar absorptivity: liters cm.$^{-1}$ moles$^{-1}$, not corrected for volume change. Each value is the mean of 12 single measurements.

## Ultraviolet Spectral Region Studies

Solutions of all the commercial and recrystallized cholesterol products in methanol (Eastman, Spectro. Grade) were prepared. Transmittance-wave length curves are shown for Armour Cholesterol and 7-keto cholesterol acetate (Steraloids) methanol solutions in Fig. 3. The shape of the curves for the cholesterol preparations were all similar.

As the absorbance values for equimolar solutions decreased at 235 mμ with recrystallizations the change was taken as a measurement for impurities. In order to estimate the amount of impurity in each cholesterol preparation 7-ketocholesterol acetate was chosen as a standard. As the free sterol, 7-ketocholesterol, is an oxidation product of cholesterol, the use of the acetate which was available commercially was felt to be justifiable. The 7-ketocholesterol acetate in methanol solution did show a linear relationship between concentration and absorbance. Table 4 shows the estimate of the cholesterol preparations purity in terms of 7-ketocholesterol acetate.

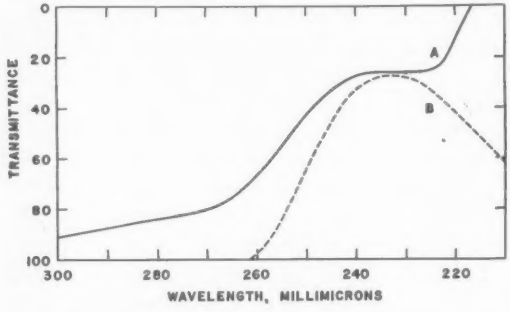


**Fig. 3.** Transmittance-wavelength spectral curves: **A.** Armour cholesterol, 450 mg. per 100 ml. methanol. **B.** 7-ketocholesterol acetate, 1 mg. per 100 ml. methanol.

**Table 4.** Purity of Cholesterol in Terms of 7-Keto Cholesterol Acetate

| *Commercial product* | *Original* (%*) | *EtOH 1X* (%*) | *EtOH 2X* (%*) | *HAc 1X* (%*) | *HAc 2X* (%*) | *DiBr 1X* (%*) | *DiBr 2X* (%*) |
|---|---|---|---|---|---|---|---|
| Armour Lot V 31105 | 99.5 | 99.8 | 99.9 | 99.9 | 99.9 | 99.9 | 99.9 |
| Pfanstiehl Lot 4907 | 99.0 | 99.5 | 99.8 | 99.7 | 99.9 | 99.9 | 99.9 |
| Steraloid Batch 4907 | 99.5 | 99.7 | 99.9 | 99.7 | 99.9 | 99.8 | 99.9 |
| K & K Lot 5550 L | 99.4 | 99.7 | — | 99.9 | — | 99.9 | — |
| General Biochemicals 6071 I | 98.0 | 98.7 | — | 99.7 | — | 99.7 | — |
| Mixture | 94.2 | 98.4 | — | 98.4 | — | 99.5 | — |

*Grams of cholesterol per 100 gm. of cholesterol plus impurities (grams of 7-ketocholesterol acetate).

## Discussion

The data shown in Table 2 indicate that the melting points generally increase with recrystallization. The original Armour, Pfanstiehl, and Steraloid cholesterol products show similar melting point ranges, while the K & K product shows a wider melting point range than the former products. The General Biochemical cholesterol melting range not only is greater than that of the other products, but the temperature values are lower than those of the other commercial products. The melting point range for the General Biochemicals cholesterol was narrowed with recrystallization. The melting point ranges do point to differences between the commercial products. Differences between recrystallized products become less apparent, however, the greater the melting point range for the original commercial products the greater the range for the recrystallized products.

The molar absorptivity values determined with both procedures, as shown in Tables 2 and 3, show an increase with recrystallization. The higher the molar absorptivity the purer the product should be. In order to determine whether the mean molar absorptivities, each of which was calculated from 12 replicate determinations, differ significantly an analysis of variance computation was made for each commercial cholesterol and its recrystallized preparations. An example of an analysis of variance computation for Armour cholesterol is shown in Table 5. As the F ratio of 3.3954 exceeds the F ratio for the 99% significance level it can be said that there is a significant difference between the molar absorptivities, as determined with the Liebermann-Burchard procedure, of the Armour cholesterol and its recrystallized preparations. Table 6 shows the results for all the commercial products and for both procedures. The data indicate that with the Liebermann-Burchard procedure the mean molar absorptivities are shown to be significantly different for 3 out of 5 commercial prod-

**Table 5.** Analysis of Variance for Armour Cholesterol Molar Absorptivity, as Determined with the Liebermann-Burchard Reaction

| *Source of estimate* | *Sum of squares* | *D. F.* | *Variance* |
|---|---|---|---|
| Within materials | 69,934 | 77 | 908.234 |
| Between materials | 18,499 | 6 | 3083.167 |
| Total | 88,433 | 83 | — |

$$F = \frac{3083.167}{908.234} = 3.3954 \qquad F_{6,77,0.01} < 3.12$$

**Table 6.** Results of Analysis of Variance between Original Cholesterol and Recrystallized Cholesterol Products and the Standard Deviation of Each Molar Absorptivity Mean

| | *Liebermann-Burchard* | | *Sulfuric acid-iron* | |
|---|---|---|---|---|
| *Commercial product* | *Significance* | *S.D.* | *Significance* | *S.D.* |
| Armour Lot V 31105 | Significant | 30 | Not significant | 200 |
| Pfanstiehl Lot 4907 | Significant | 30 | Significant | 100 |
| Steraloid Batch 4907 | Not significant | 30 | Significant | 100 |
| K & K Lot 5550 L | Significant at 95% level | 30 | Significant | 200 |
| General Biochemicals 6071 I | Significant | 20 | Significant | 200 |
| Mixture | Significant | 20 | Significant | 200 |

ucts. The data calculated for the sulfuric acid-iron procedure indicate that the mean molar absorptivity values were significantly different for 4 out of 5 commercial products.

The estimates of cholesterol purity measured in terms of 7-ketocholesterol acetate are shown in Table 4. When the estimate of the original commercial cholesterol purity was less than 99.4% the analysis of variance results show that both the Liebermann-Burchard and the sulfuric acid-iron procedures can detect significant differences between molar absorptivity values for the original and recrystallized cholesterol preparations. With purity estimates above 99.4% the sulfuric acid-iron procedure was sensitive enough to detect differences due to recrystallization for the Steraloid and the K & K cholesterol preparations. The ratio of the molar absorptivity values for the sulfuric acid-iron procedure to the Liebermann-Burchard procedure is about 6.5, indicating the former method to be a more sensitive method than the Liebermann-Burchard method for the analysis of cholesterol.

The standard deviations which are pooled estimates for the precision of the measurements for each commercial cholesterol and its recrystallized preparations are shown in Table 6. The coefficient of variation (100 $\times$ S.D./mean molar absorptivity), for both methods, shows that for 68% of the measurements for one material the variation in precision is within 2%. It can also be seen that the standard deviations may be affected by the materials being measured.

Comparison of the mean molar absorptivity values for 1- and 2-times-recrystallized cholesterol preparations by means of the statistical "t-test" did not show any significant differences between the preparations. Here, again, the standard deviation around the mean molar absorptivity value was significantly greater than the difference between the 1- and 2-times-recrystallized cholesterol molar absorptivi-

**Table 7.** Results of Analysis of Variance between the Molar Absorptivities of the Cholesterol Preparations from Different Commercial Sources

| *Cholesterol preparation* | *Liebermann-Burchard* | *Sulphuric acid-iron* |
|---|---|---|
| Original | Significant | Significant |
| EtOH 1X | Significant | Not significant |
| HAc 1X | Not significant | Significant |
| DiBr 1X | Significant | Significant |

ties, indicating again that the precision of the methods is not good enough to detect small differences. It is for this reason that 2-times-recrystallized cholesterol was not prepared for all commercial products.

Analysis of variance computations were made to determine whether the commercial cholesterol products differed significantly between companies or the similarly recrystallized preparations differed significantly because of their origins. The results are shown in Table 7. Both methods show significant differences between the molar absorptivities of the original cholesterol preparations. This is to be expected, as the purity of the original preparations were shown to differ widely by the melting point determinations and the ultraviolet spectral studies. However, the results shown for the ethyl alcohol and acetic acid recrystallized cholesterol preparations show no conclusive results, as the molar absorptivities are significantly different for one method or the other. According to the results, the dibromide derivative recrystallization method cholesterol preparations show significant differences of the molar absorptivity values for both methods. While specific impurities in each cholesterol could be influencing the results it is more probable that the variability of the methods could explain inconclusive results.

## Conclusions

The dibromide method is recommended for cholesterol recrystallization as the results show that the materials obtained have greater molar absorptivities than the materials obtained with the classic ethyl alcohol or the acetic acid recrystallization methods. An example of the effect of no recrystallization may be shown by the molar absorptivity values for General Biochemicals cholesterol. There is an approximately 6% difference between the molar absorptivities, for both methods, between the commercial and the dibromide recrystallized preparations. This would lead to a 6% error in concentration if the commer-

cial product was used as a standard. Another example of an error due to the use of impure cholesterol for a standard is shown by our molar absorptivity measurements of a 1 ml. = 2 mg. cholesterol in glacial acetic acid Harleco standard solution. There would be an approximately 4% concentration error with the Liebermann-Burchard procedure and a 14% concentration error with the sulfuric acid-iron method.

In order to check cholesterol preparations for use as standards it is recommended that the molar absorptivity values of 1750 ± 30 (1 S.D.) for the Liebermann-Burchard procedure, as described, and 11,500 ± 100 (1 S.D.) for the sulfuric acid-iron procedure should be used as a guide to ascertain purity.

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# Acrylamide-Gel Electrophoresis of Hemoglobins

*Masumi Nakamichi and Samuel Raymond*

**Details of a technic for analysis of hemoglobin mixtures using acrylamide-gel electrophoresis are presented. The method offers increased speed and reproducibility. Measurements on the unstained gel are made at 525 and at 420 mμ in a procedure affording increased precision of measurements for minor components. The gel is then stained with amidoblack to detect and measure nonhemoglobin components. The major component of normal human adult hemoglobin is separated into two components in this medium. The two components ($A_1$, $A_3$) appear in varying relative amounts depending on the source of the specimen. The normal minor component $A_2$ also appears. After staining the pattern with a protein dye such as amidoblack 10B, additional components appear in the electrophoresis patterns of hemolysates from carefully washed red cells. These components have a constant migration ratio referred to hemoglobin $A_1$ and appear to be nonheme-containing intraerythrocytic proteins.**

SINCE the discovery of abnormal hemoglobin by Pauling *et al.* (*1*), many different technics have been used for the separation and identification of hemoglobins, e.g., paper electrophoresis (*2*), column chromatography (*3*), and starch-gel electrophoresis (*4, 5*). We have investigated acrylamide-gel electrophoresis for the analytic separation of approximately 1250 hemoglobin samples. On the basis of this experience, we have evolved a convenient, rapid, and reproducible technic for this analysis and have improved the precision of measurement of the minor components.

Raymond and Wang (*6*) described acrylamide gel as a convenient and reproducible medium for gel electrophoresis. Among other ad-

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vantages, it requires neither preliminary hydrolysis nor heating in its preparation. Its transparency permits the direct measurement of hemoglobin patterns by transmission densitometry. In this paper we show that minor components of a hemoglobin pattern can be measured in the near ultraviolet and major components in the red band, to increase the relative precision of the former (See Appendix). These measurements are made on the unstained pattern, which can then be dyed with amidoblack to permit measurement of nonheme proteins, which are invariably present in small amounts. We also present data on the relative migration rates of the various hemoglobins and nonheme proteins which have been encountered in our laboratory. Finally, we present a brief discussion of the application of this material to clinical diagnosis.

## Solutions and Reagents

The reagents, including Tris-EDTA-borate buffer, dye solution, washing solution, and acrylamide monomer solution were the same as used previously (*6, 7*). The gelling procedure was the same. It was found most convenient to prepare the 5% acrylamide solution in large lots (up to 4 L. in buffer) adding 2 ml. of dimethylaminopropionitrile per liter of solution. This solution keeps at least a month under refrigeration with no noticeable change but must be warmed to room temperature before adding the catalyst (0.2 gm. ammonium persulfate per 100 ml.) to avoid the formation of minute bubbles.

## Apparatus

The apparatus used previously (*6*) included a separate container to mold a gel slab of uniform thickness and a horizontal cooling-plate cell for the electrophoresis of the sample in the prepared gel. The present work employs a vertical-gel-electrophoresis apparatus combining the mold and the electrophoresis cell in one convenient unit. This apparatus (*7*) molds the gel slab between two cooling plates in a horizontal position. The assembly is then placed in a vertical position for the electrophoresis. Smithies (*8*) has pointed out the advantages obtainable by vertical electrophoresis in starch gel.

In the present apparatus, buffer reservoirs at the upper and lower ends of the cooling plates make direct contact with the ends of the gel slab without the intervention of wicks. Hemoglobin samples of 5–10% concentrations are pipetted directly into preformed slots in the upper

edge of the gel. Detailed directions for the operation of the apparatus are available from the manufacturer.*

## Procedure

### Preparation of the Hemoglobin Samples

Hemoglobin solutions were prepared by a procedure modified from Drabkin (*9*). Collect 5 ml. of whole blood in a tube containing sodium oxalate as an anticoagulant, remove the plasma, and wash the red cells four times with physiologic saline solution. Lyse the packed red cells by shaking for 5 min. with 1–2 volumes of distilled water and 1 ml. of toluene. Centrifuge the mixture, remove the supernatant toluene layer by pipetting, and remove the stroma mass by filtering through a moistened Whatman No. 1 filter paper. The final concentration of the hemoglobin is about 8–10 gm./100 ml. For storage, saturate the hemoglobin solution with carbon monoxide gas and freeze in 1-ml. tubes. Old samples of hemolysates tend to streak on the gel. This tendency may be removed by repeating the toluene procedure.

### Application of the Sample

Using a Sahli or other capillary-tip pipet, apply 0.02 ml. of the hemoglobin solution to the gel slots by holding the tip of the pipet below the surface of the buffer, and 2–4 mm. above the slot, and allowing the hemoglobin solution to drain slowly into the slot to form a layer within the slot.

For most precise quantitative data, make an electrophoresis prerun without sample applied for a period of 1 hour, using a current of 180 ma. at about 300 v. Then apply the sample and allow it to equilibrate for 1 hour before restarting the electrophoresis. These equilibration periods stabilize the gel–buffer–sample system without external dialysis and produce the most reproducible data. For qualitative analysis of hemoglobin mixtures for clinical diagnosis, the equilibration periods can be omitted entirely with excellent resolution and adequate reproducibility of patterns.

### Conditions for Electrophoresis

With the apparatus employing tap-water cooling, 20 v per centimeter can be applied to the gel, producing migration of hemoglobin

*We thank the manufacturer, E-C Apparatus Corp., Swarthmore, Pa., for kindly supplying the apparatus used in the work.

A a distance of 10 cm. in 3 hours. This requires the application of 300 v at 140–160 ma. to the cell. Although higher voltages (up to 800 v at 300 ma.) can be applied to the cell without overheating, the reduction in running time does not justify the extra effort required. Conversely, it is certainly worthwhile to reduce the time required from 18–24 hours as in the older methods to the 3 hours used in the present one.

### Measurement of Hemoglobin Patterns

Depending on the purpose of the analysis, one of three types of pattern evaluation may be used. The simplest is a visual comparison of the hemoglobin bands in the unknown sample with a standard normal hemoglobin pattern. Visual comparison can be used to establish the presence or absence of abnormal hemoglobins for clinical diagnostic purposes. With some experience, one can detect the presence of elevated F or $A_2$ to assist in the diagnosis of thalassemia. Each visible component can be graded in intensity on any convenient scale and its distance of migration from the origin recorded as a ratio to the migration distance of a standard hemoglobin run at the same time under identical conditions.

More accurate quantitation of hemoglobin mixtures is based on measurement of the pattern, preferably using a photoelectric densitometer. Automatic recording of the optical density over the hemoglobin pattern eliminates much tedious recording and plotting of data, and the areas of the individual peaks can be integrated to provide data from which percentage composition of the hemoglobin mixture can be calculated. For the densitometer and recording apparatus used, it is necessary to establish proportionality of peak area to quantity of hemoglobin present. We tested several densitometer–recorder combinations and found the Photovolt Model 525 densitometer with the model 43 Varicord Linear/Log recorder to give the most reliable results.

Using 0.02 ml. of 5–10% hemoglobin solution in a gel slot 2 cm. wide as described above, a hemoglobin pattern consists of one or two major peaks within the proportionality range of the densitometer–recorder when measured in the unstained state with a red filter. The major peaks may show shoulders or partly separated peaks on either side, contributing to the size of the major peaks.

The minor components which are always present in hemolysate do not show up well using a red filter, and if identifiable at all on the recorded pattern, cannot be measured with precision because the area of the peak is less than the lower limit of proportionality. This dif-

ficulty can be overcome by rerecording the hemoglobin pattern using a near ultraviolet filter. Because of the sharp absorption band of hemoglobin pigments in the near-ultraviolet, the so-called Soret band, the second recording magnifies the peaks of the minor components, bringing them into the proportionality region of recording, but at the same time throws the major peaks far beyond the upper limits of proportionality. Since the red filter recording and the ultraviolet filter recording differ only by a factor of proportionality (See Appendix), it is possible to measure areas of minor peaks on the ultraviolet recording and, after multiplying by a suitable factor, to compare them with areas measured on the red recording. The comparison measured is more precise because of the increased areas measured.

Densitometer recording the unstained gel, whether in the red or in the ultraviolet region, does not reveal the full information available in these electrophoresis patterns. There are one or two additional minor components present which do not contain hemoglobin pigments. These are revealed by staining the gel with 1% amidoblack and decolorizing with dilute acetic acid as previously described (*6*). The nature and significance of these minor nonhemoglobin components are as yet unknown.

To sum up, the complete recording of a hemoglobin pattern in our method comprises these parts: First, recording the complete pattern using a red filter; second, rerecording using an ultraviolet filter; and third, following staining with amidoblack and clearing, a final recording with no filter. For storage, the gel strips are wrapped in a vapor-barrier plastic film (Saran Wrap).

## Results

More than 500 hemoglobin samples (over 1300 determinations) were studied. The patterns obtained from normal erythrocytes and from the erythrocytes of patients with various hemoglobinopathies were examined three times in the densitometer: first using a 525-m$\mu$ filter, second using a 420-m$\mu$ filter, and finally as the amidoblack-stained pattern without the use of a filter. The order of mobility in acrylamide gel of the several hemoglobins is (origin) X, C, $A_2$, S, F, $A_1$ (fastest). We did not encounter in our series any of the hemoglobins known to migrate faster than Hemoglobin $A_1$ in starch gel (Fig. 1, p. 140, and Table 1, p. 143).

In acrylamide gel a change in *p*H over the range 9.0–9.5 altered the mobilities of various hemoglobins only slightly. The buffer *p*H was

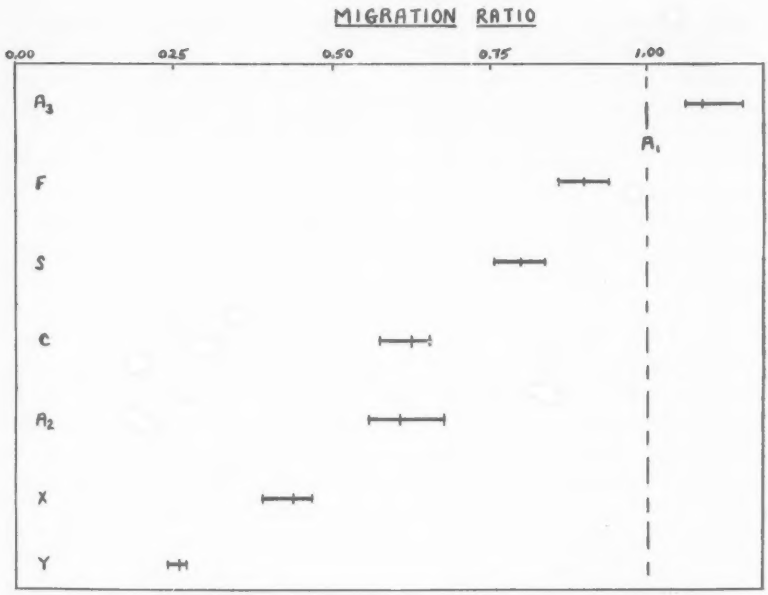


**Fig. 1.** Migration ratio of abnormal hemoglobins relative to hemoglobin $A_1$. Each bar shows mean value and range of migration ratio measured from site of application of sample to peak absorption of hemoglobin zone.

therefore chosen to be 9.2–9.3, the midpoint of this range, to minimize the effect of accidental *p*H variations on the patterns obtained. Adequate mobility differences were obtained, permitting good resolution of all types of hemoglobins available to us except $A_1$-F, and $A_2$-C. These two pairs could be distinguished by other known criteria.

### Normal

In 275 normal unstained patterns, two components were detected. On the basis of their absorption in the Soret region, these were hemoglobin components designated as $A_2$ and $A_1$. The $A_2$ component migrated a distance of 0.61 (range, 0.56–0.68) relative to the major component $A_1$.

Many samples of presumably normal hemoglobin exhibited a shoulder or a separate peak on the leading edge of the main component. The additional component indicated thereby has been designated $A_3$ (migration ratio, 1.09). The nature of this component is not firmly

established, but it has been suggested (*10*) that $A_3$ observed in other electrophoresis media is the result of chemical alteration of $A_1$. We have observed similar shoulders and leading peaks in the major component of hemoglobin C, but not on hemoglobin S.

Hemoglobin F, when present in sickle thalessemia samples, forms a shoulder or peak on the leading edge of the S component and contributes to the size of the S peak. It is not feasible to quantitate the proportion of F present from these patterns at *p*H 9.2

In the stained pattern a nonpigmented component X appeared which migrated a distance of 0.435 relative to $A_1$. A second nonhemoglobin component Y (migration ratio, 0.261) was also often observed in a concentration lower than that of X. Neither of these components corresponded in its position with any clearly defined band in the serum patterns.

#### Sickle Trait

This was diagnosed in 43 patients. The bands identified in the pattern before dyeing were $A_2$, S, $A_1$, and $A_3$, in order of their migration. Band X appeared on staining.

#### Hemoglobin S Trait

In samples from 20 patients, the bands identified in the unstained pattern were C, $A_1$, and $A_3$. Band X appeared on staining.

#### Hemoglobin C–Sickle-Cell Disease

In samples from 10 patients, the bands identified were C and S. Band X appeared on staining.

#### Thalassemia

In samples from 17 patients, the bands identified were $A_2$, $A_1$, and $A_3$. Band X appeared on staining.

#### Sickle–Thalassemia

In samples from 4 patients, the bands identified were $A_2$, S, and F. Band X appeared on staining.

To the clinician, the diagnosis of thalassemia is a problem of great interest. The diagnosis of the "classic" type of thalassemia (*10*) is suggested by the elevation in the electrophoretically separable component $A_2$. A previous technic using starch gel (*4*) gave an average of 3.6% $A_2$ for 29 normal individuals (range, 2.8–4.5%) and an average of 6.8% $A_2$ (range, 5.2–8.4%) for 17 patients diagnosed as thalassemias. Other technics including starch block (*11*) and paper elec-

trophoresis (*12*) gave average values ranging from 2.6–3.5% $A_2$ for normal individuals. For thalassemic patients, the values of $A_2$ were stated as "increased" and 9% $A_2$, respectively.

Our results in acrylamide gel averaged 2.6% $A_2$ (range, 0.5–5.0%) for 106 determinations in nonthalassemic individuals and 7.4% $A_2$ (range, 5.1–15.8%) fof 17 thalassemic patients. Until an absolute method for the determination of $A_2$ hemoglobin becomes available, these values must be regarded as only relative; we think they are sufficiently precise and reproducible to be relied upon for clinical diagnosis. In our series none of the normal individuals had an elevated $A_2$, nor did the clinically diagnosed thalassemics have an $A_2$ concentration in the normal range. The two ranges, however, met exactly at 5.0–5.1%, and it is likely that in a larger series some overlap might be found.

## Appendix

The amount of light transmitted by a filter at any wavelength is given by the expression

$$I = t_f I_o \tag{1}$$

in which $I_o$ is the intensity of the source, and $t_f$ is defined as the transmission factor of the filter and is a function of wavelength. The total amount $I_f$ of light transmitted over a band of wavelength from $\lambda_1$ to $\lambda_2$ is therefore

$$I_f = I_o \int_{\lambda 1}^{\lambda 2} t_f \, d\lambda \tag{2}$$

If a unit-length cuvet containing a light-absorbing solution is placed in the optical path, the intensity transmitted is reduced further by the factor $e^{-ac}$, where $c$ is the concentration and $a$ is the absorbancy. The factor $a$ is again a function of wavelength but is considered independent of the concentration (Beer's law). The transmitted intensity $I_t$ than the solution is therefore

$$I_t = I_o e^{-c} \int_{\lambda 1}^{\lambda 2} e^{-a} t_f \, d\lambda \tag{3}$$

The apparent optical density of the solution in the cuvet is the logarithm of $I_f/I_t$

$$A = c \left[ \log \int_{\lambda 2}^{\lambda 1} t_f \, d\lambda - \log \int_{\lambda 1}^{\lambda 2} e^{-a} t_f \, d\lambda \right] \tag{4}$$

Expression 4 reduces to Beer's law

$$A = ca \tag{5}$$

only if $t_f$ and $a$ are constant over the wavelength interval employed. For any given filter, however, the expression in brackets is a constant which is just as characteristic of the individual hemoglobin component as the absorbancy is. We may therefore rewrite Equation 4 as

$$A = c\,K_f, \tag{6}$$

defining the bracketed expression as $K_f$ to denote that it is a constant depending on the filter as well as on the absorbing compound. When filters centering at 420 and 525 m$\mu$ are used as in our procedure above, the ratio of optical densities

$$\frac{(\text{O.D.})_{420}}{(\text{O.D.})_{525}} = \frac{\text{K}_{420}}{\text{K}_{525}} \tag{7}$$

is a constant since both $K_{420}$ and $K_{525}$ are constants. This ratio cannot in general be calculated from the molar absorbancies at the peak wavelength used but must be determined experimentally. In hemoglobin analysis by transmission densitometry of acrylamide-gel patterns, the ratio of $K_{420}$ and $K_{525}$ is approximately 5 (Table 1), indicating a fivefold increase in precision of the measurement.

Experimentally, hemoglobin samples containing unknown amounts of $A_2$ hemoglobin were applied to acrylamide gel in the vertical-gel-

**Table 1.** Filter-factor Determination

| *425 filter area* | *520 filter area* | *Ratio 425/520* |
|---|---|---|
| 246 | 52 | 4.7 |
| 223 | 49 | 4.6 |
| 219 | 50 | 4.4 |
| 223 | 56 | 4.0 |
| 207 | 43 | 4.8 |
| 198 | 47 | 4.2 |
| 189 | 41 | 4.6 |
| 210 | 44 | 4.8 |
| 171 | 46 | 3.7 |
| 125 | 37 | 3.4 |
| 121 | 24 | 5.0 |
| 184 | 33 | 5.6 |
| 187 | 35 | 5.3 |
| 185 | 34 | 5.4 |
| 174 | 30 | 5.8 |
| 164 | 29 | 5.7 |
| 183 | 32 | 5.7 |
| 227 | 37 | 6.1 |
| | | AV. 4.9 ± .6 |

**Table 2.** MIGRATION RATIO

| *Hemoglobin* | *No. of determinations* | *Migration ratio* | *Range* |
|---|---|---|---|
| $A_3$ | 193 | 1.09 | 1.06–1.15 |
| $A_1$ | | 1.000 | |
| $A_2$ | 295 | 0.61 | 0.56–0.68 |
| S | 62 | 0.80 | 0.76–0.84 |
| C | 22 | 0.63 | 0.58–0.66 |
| F | 27 | 0.90 | 0.86–0.94 |
| X* | 42 | 0.44 | 0.39–0.47 |
| Y* | 9 | 0.26 | 0.24–0.27 |

*X and Y are nonhemoglobin fractions which appeared on staining.

electrophoresis apparatus of standard design. Following electrophoresis under standard conditions of temperature, voltage, and current, the patterns were measured directly in a specially equipped Photovolt densitometer with recorder, using filters of wavelength 420 and 525 mμ. The patterns were not stained or treated in any way prior to measurement.

The quantities of hemoglobin applied to the gel were such as to give areas of the $A_2$ peak in the range of 30–60 integration units (equal to 0.3–0.6 sq. in.) which could be measured with a precision of 3–5% when using the 420 filter. As expected, the same areas measured in the visible region using 525 filter gave areas of 3–10 units, which could be in error by as much as 50%. (The unit is an arbitrary measure of area determined with a planimeter.)

The $A_1$ component measured in the same way and at the same time gave areas in the visible region of 100–200 integration units. In the near ultraviolet the $A_1$ areas were so large as to run off scale on the densitometer used, but by repeating the measurements with proper adjustment of the range control switch, the upper ends of the peak areas could be brought on scale. We were, therefore, able to determine the ration of $A_1$ peak areas at two wavelengths. Assuming that the previous equations take the same form for both $A_1$ hemoglobin and $A_2$ hemoglobin, we can employ the experimentally determined ratio for $A_1$ to obtain a reasonably precise value for $A_2$ in the 525 region by multiplying the measured area of $A_2$ in the 420 region by the experimentally determined ratio for $A_1$.

The areas found with 525 filter have been compared with the areas found with the 420 filter for a number of samples (Table 1). The average ratio is 4.9.

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# Extraction Procedure for the Measurement of Blood Volume with Brilliant Vital Red

*Y. Takeda**

**A simple extraction method usable for Brilliant Vital Red is described. The dye is displaced from its combination with albumin by detergent, adsorbed on a cotton cellulose column, and eluted with 40% acetone–water at *p*H 8.0. Recovery of Brilliant Vital Red from plasma averaged 95%. Measurements are not affected when the plasma samples are hemolyzed, jaundiced, or lipemic, or when dextran is present. Patients therefore require no previous preparation. Simultaneous measurements of blood volume were made by Brilliant Vital Red and $I^{131}$–albumin in 17 patients. The results of the two methods were in close agreement.**

A METHOD has been reported for extracting T-1824 (Evans Blue) from plasma on a cellulose column (*1*). T-1824 is structurally closely related to Brilliant Vital Red, a dye used earlier for the measurement of plasma volume (*2*) but abandoned because of the errors caused by dissolved hemoglobin in the plasma and for other reasons (*3–5*). Congo Red, now chiefly used as a test for the presence of amyloidosis (*6*), is also structurally closely related to T-1824. Red dyes, other things being equal, have a slight advantage over blue dyes, in that any tint appearing in the skin, more noticeable in pale subjects after repeated injections of dye, is faintly pink rather than faintly blue, simulating cyanosis. It thus seemed of interest to see if the cellulose extraction method could be adapted satisfactorily to the extraction of Brilliant Vital Red. This was found to be possible. Studies were therefore made to test the validity of the results of measurements of plasma volume by Brilliant Vital Red using the extraction method simultaneously with an $I^{131}$–albumin method in a series of patients. Agreement was satisfactory.

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## Observations with Brilliant Vital Red

### Materials and Methods

#### Purification of Brilliant Vital Red

The Brilliant Vital Red* contained considerable amounts of an orange-colored impurity. The dye was purified according to the method of Fieser (*7*) as follows: Five grams were dissolved in 48 ml. of distilled water and heated to 85° in a water bath. Then 13.50 gm. of sodium acetate ($CH_3 \cdot COONa \cdot 3H_2O$) were added slowly in 4–5 portions to the hot solution with vigorous stirring. The mixture was filtered, while hot, on a Buchner funnel using two pieces of Whatman filter paper No. 41. The filter cake was removed from the filter papers and redissolved in 48 ml. of distilled water. The solution was reheated to 85°, and the dye was again salted out with the same amount of sodium acetate. The salting-out procedure was repeated two more times, which removed all visible traces of the orange impurity. After drying at 110° in an oven for about 24 hr., the product was ground fine. The sodium acetate in the powder was removed by repeated extraction with hot 95% ethanol. For this the powder was transferred to a beaker, 20 ml. of near-boiling 95% ethanol was added, and the mixture was placed in a water bath at 85° and stirred gently for 10–15 min. The ethanol was then decanted. This extraction process was repeated 6–7 times till the addition of 3 drops of concentrated sulfuric acid to 10 ml. of the supernatant, cooled in ice water, showed no precipitate, indicating that less than 3 mg. of sodium acetate per 10 ml. supernatant was present. Finally the dye was dried in an incubator and then ground fine. A 1% solution of the dye was prepared in sterile pyrogen-free water, and 5 ml. of this was sealed in ampules and autoclaved for 1 hr. at 20 lb. pressure per square inch. Figure 1 shows the absorption spectrum of the dye thus purified, determined with the Beckman DU spectrophotometer. The peak absorbance is at 505 m$\mu$. The chemical formula of the dye is identical with that of the dye used by Hooper *et al.* (*2*).

#### Principle

One to three milliliters of dyed plasma are reacted with a detergent (Teepol†) in a glycine-HCl-buffer. The mixture is poured on to a cotton cellulose column to the top of which the dye adsorbs. The column

*Lot No. 14634 obtained from the National Aniline Division, the Allied Chemical & Dye Corporation, New York, N. Y.

†Teepol 610 Concentrate, Shell Chemical Co., New York 17, N. Y.

is washed free of substances that interfere with accurate spectrophotometry with citrate–phosphate buffer, and the Brilliant Vital Red is eluted with 40% acetone-water at *p*H 8.0. The eluted dye is buffered to *p*H 6.5 and the absorbance is measured in a spectrophotometer.

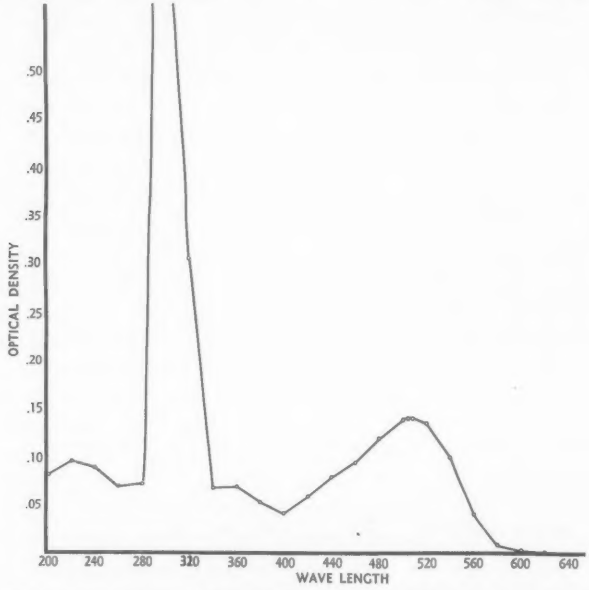


**Fig. 1.** Absorption spectrum of Brilliant Vital Red in buffered acetone water. Measurements were made in a Beckman DU spectrophotometer on a solution of 2.5 mg. purified Brilliant Vital Red dissolved in 350 ml. of *p*H 6.5 citrate–phosphate buffer (300 ml. Reagent 2, 50 ml. of Reagent 4) and made to 1 L. with *p*H 8.0 acetone water (Reagent 3).

### Reagents

1. *Glycine-HCl-Teepol.* Fifty milliliters of 0.2M glycine (1.5 gm./100 ml.) is mixed with 11.4 ml. of 0.2N HCl (1.92 ml. conc. HCl to 100 ml. $H_2O$). Ten milliliters of Teepol is added, and the mixture is made to 200 ml. with distilled water. The mixture is not very stable and therefore should be freshly prepared every day.

2. *Citrate-Phosphate buffer, pH 7.0.* Mix 6.5 ml. of 0.1M citric acid (21.0 gm. of $H_3C_6H_5O_7 \cdot H_2O$ per 1000 ml.) and 43.6 ml. of 0.2M sodium phosphate (71.7 gm. of $Na_2HPO_4 \cdot 12H_2O$ per 1000 ml.) and make to 100 ml. with distilled water. This keeps for about 7 days under refrigeration.

3. *Acetone–Water, 40%, pH 8.0.* Four parts of acetone are mixed with six parts of water. On the day of use adjust the required volume to *p*H 8.0 with 1N NaOH using *p*H paper. For the preparation of a 250-ml. volume, 0.65–0.7 ml. of 1N NaOH is required. Discard after 1 day's use.

4. *Citric acid, 0.16M.* 16.80 gm. citric acid is made to 500 ml. with distilled water. This is stable over several weeks under refrigeration.

5. *Cellulose Suspension.* Suspend just before use one of Whatman's Ashless Tablets* in about 40 ml. of glycine–HCl–Teepol mixture. Shake and stir thoroughly to form an even cellulose suspension. This should be discarded after 1 day's use.

6. *Standard Dye Solution.* Dilute accurately 0.500 ml. of the 1% stock solution of Brilliant Vital Red to 100.0 ml. with distilled water and refrigerate.

#### Preparation of Column

One column is required for the standard, one for each of the plasma samples analyzed, and one for the blank plasma sample.

1. Pyrex† glass wool is taken up in forceps and inserted into the top of the column holder shown in Fig. 2. Distilled water is poured into the tube as lubricant, and the glass wool is firmly packed above the small constriction with a long glass rod to give a column about 5 mm. high. About 0.1 gm. glass wool is required. Beakers to collect the outflow are placed beneath each column.

2. Using a 10-ml. graduated pipet with a cut-off tip, transfer about 10 ml. of the cellulose suspension (Reagent 5) into the top of the column holder. Let the suspending fluid drain out so that an even column of the cellulose, 4–5 cm. high, is formed on top of the glass wool. The height of the column may vary between 4 and 6 cm. without causing trouble.

3. Wash each column with 10 ml. of glycine–HCl–Teepol, limiting the rate of outflow with a 22-gauge lever needle (4–5 ml. per minute).

#### Preparation of the Plasma Sample and Standard

1. Two milliliters of the dyed plasma samples, the standard plasma, and the blank plasma, are pipetted into the requisite number of 50-ml. Erlenmeyer flasks. (Any volume of plasma from 1 to 3 ml. may be used, but for calculation of the results, it is easiest to use the same volume

*Whatman Ashless Tablets, obtained from W & R Balston Ltd., England.
†Corning Glass Works, New York, N. Y.

of plasma for each sample, standard, and blank.) Either plasma or serum may be used, but heparinized plasma is the most convenient.

2. Both the standard plasma and the blank plasma are obtained from a sample of the patient's blood drawn just prior to the dye injec-

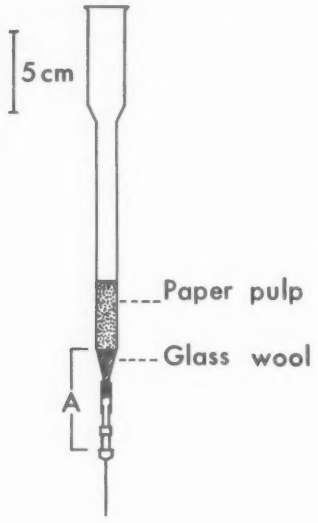


**Fig. 2.** Chromatographic column holder. Sufficient glass wool is used to prevent small particles of cellulose passing through and blocking hypodermic needle which controls outflow rate. This needle is attached to tip of column holder by a short piece of transparent tubing. Disposable plastic needle adaptors supplied with transfusion sets provide good attachments if shortened. Dead space between bottom of the column and butt of needle, marked *A*, must be kept to a minimum.

tion. To prepare the standard dyed plasma, exactly 0.500 ml. of the standard 1:200 Brilliant Vital Red solution (Reagent 6) is added to 2 ml. of the standard plasma in the 50-ml. Erlenmeyer flask; the two are mixed thoroughly by gently swirling and then allowed to stand for 2 min. A standard dyed plasma is always prepared to correct for any small quantity of colored impurities in the dye and as an internal check on the method.

3. Fifteen milliliters of glycine–HCl–Teepol are added to each Erlenmeyer flask. The contents are mixed thoroughly by gentle swirling for 15 sec.

#### Dye Extraction

1. The contents of each flask are poured gently on to cellulose columns. During these and subsequent additions the fluids poured into the column containers should disturb the top of the columns as little as possible. Each flask is washed out with 5 ml. of glycine–HCl–Teepol,

and when most of the fluid has run through the column, the washings are poured on to the appropriate column. Finally, when the washings have almost run through, 10 ml. of citrate–phosphate buffer is poured on the top of the column. The dyes should be held as a red band in the top 10 mm. of the column, and after the final citrate–phosphate wash, the remainder of the column should be perfectly white. If not, this indicates that the citrate–phosphate washes were not sufficient and more is required. During the fixation of the dye to the cellulose, flow through the column should be about 1 ml. a minute.

2. As soon as the final wash has flowed down to the top of the column, a 10-ml. volumetric flask containing 0.5 ml. of 0.16M citric acid is placed under the delivery needle, and approximately 5 ml. of *p*H 8.0 acetone–water (Reagent 3) is poured on the top of the column. In 10 min. a second portion of 4–5 ml. *p*H 8.0 acetone–water is poured on the top of the column, and the eluate is collected until the flask is filled almost to the 10-ml. mark. The volume is then brought to the mark with acetone–water, the contents of the flask are mixed, and the flasks are stoppered. Flow through the column during elution should be about 1 ml. per min.

#### Photometry

The absorbancies of the acetone–water extracts are measured in a spectrophotometer. With the Coleman Junior Spectrophotometer, 19-mm. circular cuvettes are used and readings are made at 505 m$\mu$. There is a linear relationship between absorbance and dye concentration over a wide range of dye concentrations. The acetone extracts should be crystal clear. If they are cloudy, this indicates that all the protein has not been washed out of the column by the citrate–phosphate buffer before the start of the elution, but this should seldom occur.

#### Calculation

The formulas for the calculation of plasma ($PV$) and blood volume ($BV$) from the data are as follows:

$$PV = \frac{V \times D \times (St - b)\ Xr}{Sa - b}$$

$$BV = \frac{\text{PV}}{1 - 0.87 \times \text{Ht.}}$$

Where $V$ indicates volume of Brilliant Vital Red solution injected, $D$, dilution of standard Brilliant Vital Red solution (400 times), $St.$, ab-

sorbance of standard, $b$, absorbance of the blank plasma. $Sa$, zero time plasma dye absorbance, $v$, volume of plasma samples analyzed. 0.87, correction factor for both trapped plasma between cells and mean "body hematocrit" (*8*), and *Ht.*, Wintrobe's hematocrit.

## Results

### The Plasma Blank

With the method described, unless dye has been injected in the preceding month, the absorbance of the blank plasma at 505 m$\mu$ in the Coleman Junior Spectrophotometer averages .005, which is less than 3% of the average absorbance seen in the dyed plasma samples of a plasma volume measurement. Studies on various abnormal plasma samples were made to determine the effects on the blank reading. Table 1 shows that the presence of lipemia, dissolved hemoglobin, and large quantities of dextran (5 gm./100 ml. plasma) were without effect on the blank reading, and with two exceptions this was also true with raised plasma bilirubin levels.

### Recovery of Brilliant Vital Red from Plasma Samples

Recovery of Brilliant Vital Red added to various plasma samples ranged between 93 and 97% and averaged about 95% (Table 2). Standard deviations of the recovery rates from clear, hemolyzed, lipemic, and jaundiced plasma and also from plasma containing dextran were 1.0, 0.6, 0.44, 1.0, and 0.47% respectively (Table 2).

### Simultaneous Measurements of Blood Volume by Brilliant Vital Red and RISA

Simultaneous measurements were made in 17 patients. After blank blood samples had been withdrawn, exactly 4 ml. of sterile purified 1%

**Table 1.** Blank Readings with Various Plasmas

| *Type of plasma* | *No. of samples* | *Mean* | *Standard deviation* |
|---|---|---|---|
| Clear plasma | 8 | .004 | .001 |
| Lipemic plasma | 8 | .003 | .002 |
| Hemolyzed plasma | 8 | .003 | .002 |
| Jaundiced plasma* | 8 | .018 | .031 |
| Dextran plasma | 8 | .004 | .001 |

*Two samples had blank readings of 0.01 and 0.1 for unknown reasons; when these are excluded, the mean absorbance was .005 ± 0.001. Two milliliters of plasma were extracted as described in the text and the measurements were made in a Beckman DU Spectrophotometer at 505 m$\mu$.

**Table 2.** RECOVERY OF BRILLIANT VITAL RED FROM VARIOUS PLASMA SAMPLES

| *Type of plasma* | *No. of samples* | *% recovery* | *Standard deviation* |
|---|---|---|---|
| Clear plasma | 14 | 95.3 | 1.0 |
| Hemolyzed plasma | 16 | 95.1 | 0.6 |
| Lipemic plasma | 8 | 95.0 | 0.44 |
| Jaundiced plasma | 8 | 94.6 | 1.0 |
| Dextran plasma | 8 | 95.1 | 0.47 |
| MEAN | 10 | 95% | 0.9% |

Two milliliters dyed plasma were extracted with 40 per cent *p*H 8.0 acetone-water. The measurements were made with a Beckman DU Spectrophotometer.

Brilliant Vital Red and exactly 4 ml. of diluted $I^{131}$–albumin solution were injected, one after the other through the same needle into the antecubital veins of the patients. After the injections, the blood was drawn back and forth in the syringe to wash out any residual dye or $I^{131}$–albumin in the syringes. Three blood samples of about 10-ml. volume were withdrawn at 15, 25, and 35 min. after the injection. The Brilliant Vital Red in the three dye-containing blood samples, the standard dyed plasma, and the blank plasma were extracted as described above. The absorbancies of the extracts from the three post-injection blood samples, corrected for the blank readings, were plotted on rectilinear graph paper to obtain by extrapolation the zero time plasma dye absorbance. The $I^{131}$–albumin measurement of plasma volume was made exactly as described by Franks and Zizza (*9*). Four lots of $I^{131}$–albumin* were used and the unbound $I^{131}$ in them averaged 1% of the total radioactivity. Agreement between the two methods was good, as shown in Table 3.

### Loss Rates of Brilliant Vital Red and RISA from the Circulation

Approximate loss rates of Brilliant Vital Red and $I^{131}$–albumin over the first 35 minutes after their injection may be calculated. There was a significant difference between the two, as shown in Table 3. The average loss rate of Brilliant Vital Red was 24.4% per hour in this group of patients. The average loss of $I^{131}$–albumin of 16% per hour is higher than that reported in normal subjects (*9*). If a value for plasma volume is required from the dye content of a single sample withdrawn during the first 10–20 min. after injection, a correction for a mean dye loss rate of 20% per hour will not lead to serious error.

*$I^{131}$–Albumin (RISA) obtained from Abbott Laboratories, North Chicago, Ill.

**Table 3.** Plasma Volume Measurements by Brilliant Vital Red and $I^{131}$–Albumin with Rates of Loss from the Plasma

| Patients | | Plasma volume (ml.) | | | Loss rates (% per hour†) | | |
|---|---|---|---|---|---|---|---|
| No. | Diagnosis | Brilliant Vital Red | $I^{131}$–albumin | % difference* | Brilliant Vital Red | $I^{131}$–albumin | % difference per hour‡ |
| 1. | Lymphosarcoma | 4031 | 4081 | −1.2 | 18 | 18 | 0. |
| 2. | Hodgkin's disease | 2508 | 2611 | −3.9 | 15.6 | 20 | − 4.4 |
| 3. | Normal | 3168 | 3070 | +2.4 | 3.96 | 3.96 | 0. |
| 4. | Myocardial infarct | 3294 | 3252 | +1.5 | 23.0 | 21.0 | + 2.0 |
| 5. | Pancytopenia | 3333 | 3317 | +0.5 | 60.0 | 39.6 | +20.4 |
| 6. | Pulmonary fibrosis | 3080 | 2900 | +6.1 | 39.6 | 30.6 | 0. |
| 7. | Polycythemia | 2746 | 2845 | −3.4 | 18.0 | 12.0 | + 6.0 |
| 8. | Polycythemia | 3907 | 3777 | +3.5 | 18.0 | 18.0 | 0. |
| 9. | Diabetes mellitus | 2826 | 2680 | +5.2 | 24.0 | 18.0 | + 6.0 |
| 10. | Pulmonary fibrosis | 2855 | 2718 | +5.1 | 14.4 | 11.2 | + 3.2 |
| 11. | Polycythemia | 2681 | 2607 | +2.8 | 30.0 | 6.0 | +24.0 |
| 12. | Paget's disease | 2250 | 2263 | +0.5 | 45.0 | 14.3 | +30.7 |
| 13. | Thyrotoxicosis | 3292 | 3155 | +5.0 | 29.4 | 13.3 | +16.1 |
| 14. | Lymphosarcoma | 2670 | 2768 | −3.5 | 27.6 | 13.1 | +14.5 |
| 15. | Duodenal ulcer | 2911 | 2838 | +2.5 | 11.0 | 8.0 | + 3.0 |
| 16. | Inguinal hernia | 2184 | 2281 | −3.9 | 18.0 | 11.0 | + 7.0 |
| 17. | Fractured leg | 2738 | 2736 | +0.06 | 19.0 | 4.2 | +14.8 |
| Means | | 2969 | 2934 | +1.13 | 24.4 | 15.94 | + 8.4 |
| | | Standard deviation = 3.3% | | | Standard deviation = 10.5% | | |
| | | $t = 1.41$ | | | $t = 3.36$ | | |
| | | $0.1 < p < 0.2$ | | | $0.01 > p > 0.001$ | | |

*Percentage difference $= \dfrac{\text{Brilliant Vital Red Plasma Volume} - I^{131}\text{–Albumin plasma volume}}{I^{131}\text{–Albumin Plasma Volume}}$

†Loss rates of Brilliant Vital Red and $I^{131}$–Albumin from the plasma as percentage per hour calculated from the plasma disappearance rates during the first 35 min. after injection.

‡Percentage difference per hour = Brilliant Vital Red Loss Rate (% per hour) − $I^{131}$–Albumin loss rate (% per hour).

## Discussion

The use of Brilliant Vital Red for the measurement of plasma volume was abandoned because of errors in its measurement caused by hemoglobin dissolved in the plasma and lipemia (*3–5*). The extraction method for Brilliant Vital Red here described eliminates these errors and makes accurate blood-volume measurements possible. The method described is simple, requires about 1 hr. for the analyses, and for routine measurements two dyed blood samples may be analyzed. If these are drawn at 10 and 20 min. after the dye injection, the mean absorbance of the two samples corrected by 1.05 will give a reasonably accu-

rate zero time plasma dye absorbance. This therefore provides an additional means of obtaining accurate measurements of plasma volume besides T-1824 and $I^{131}$–albumin, with cosmetic advantages over T-1824 and the avoidance of the injection of radioactivity.

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# Errors Associated with the Direct Measurement of Human Serum Cholesterol Using the $FeCl_3$ Color Reagent

*Robert V. Moore and Edwin Boyle, Jr.*

**Evidence is presented concerning errors in the direct determination of human serum cholesterol with a ferric-chloride color reagent. It is shown that in physiologic concentrations the vitamins A, D, E, K, and niotinic acid cause no error, but that hemoglobin, anticoagulants, and impurities in acetic acid can cause errors. The control of these errors is discussed. Errors caused by steroids, added salts, and high bilirubin content of serum are discussed.**

THERE ARE reports (*1, 2*) criticizing the direct-cholesterol method of Zlatkis *et al.* (*3*), which utilizes a ferric-chloride color reagent, for giving high values for the cholesterol concentrations in human sera. Zlatkis *et al.* warned that glyoxylic acid, which occurs in commercial acetic acid, might interfere with the test. (Glyoxylic acid reacts with tryptophan, from the protein in serum, in the presence of concentrated $H_2SO_4$, to give a color which is measurable at the wavelength used in the cholesterol procedure.) When acetic acid is refluxed with $CrO_3$, then distilled, as described by MacIntyre and Ralston (*4*), all impurities which affect this test are removed and the cholesterol values obtained using the $FeCl_3$ color reagent are comparable to values obtained using extraction followed by the Liebermann-Burchard color reagent. Henly (*5*) and Morris (*2*) using similarly distilled acetic acid confirmed this finding. These three papers, however, did not show why $CrO_3$ was necessary. Henly (*5*) also pointed out that the original color reagent of Zlatkis was unstable and that it was water sensitive. These

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objections are overcome by the use of the $FeCl_3$ color reagent modifications of Rosenthal *et al.* (*6*). The sensitivity of the $FeCl_3$ color reagent, which is greater than the Liebermann-Burchard reagent, is pointed out by Henly (*5*) and Smit and Weymeyer (*7*). This sensitivity, which magnifies small errors in technic and measurement, and errors caused by changes in initial temperatures are pointed out by Furst and Lange (*8*). Kinley and Krause (*9*) have reported that vitamin A seriously affects direct human serum cholesterol values. Hollinger *et al.* (*10*), without giving any data, claim that the $FeCl_3$ reagent of Zlatkis "... reacted with so many agents in blood serum as to preclude specificity for cholesterol."

It is the purpose of this paper to present data relative to the aforementioned criticisms as applied to human serum-cholesterol measurements using the Rosenthal color reagent with the Zlatkis procedure. The reasons for distilling and the method of distillation of acetic acid will be given. Also included are data on the effects of hemolysis, vitamins other than A, and errors caused by anticoagulants used in preparing plasma.

## Materials and Methods

All reagents and all solvents were reagent grade except the following: cholesterol, which was recrystallized until the final product had a melting point of 148–149°; vitamins A, D, E, and K, which were commercial forms; and nicotinic acid powder U.S.P.

The cholesterol procedure used was that described by Zlatkis *et al* (*3*) using the color reagent modification of Rosenthal *et al* (*6*). Essentially it consists in pipetting 0.025 ml. of serum into a suitable container* adding 3 ml. of redistilled acetic acid, then adding 2 ml. of ferric chloride color reagent down the side of the container in such a way as to layer it below the acetic acid. The container is capped and the mixture is abruptly and thoroughly mixed. After 10 min. the absorbance is read at 560 m$\mu$ in a spectrophotometer. The color is completely stable for 10 min. A standard cholesterol in acetic acid is measured daily. Cholesterol values of lipoprotein fractions obtained by preparative ultracentrifugation are measured as previously described (*11*).

Distillation of acetic acid was carried out in an all-glass apparatus using a Claisen distilling head. Distillation was started with 2 L. of acid and stopped after it was shown that the acetic acid obtained gave

*This laboratory has found the Plastainer vial (Owens-Illinois Glass Co.), 7 dram, to be a very satisfactory container as it is resistant to chemical reaction and breakage.

reproducible values, which occurred after about 1 L. of acid had been distilled. The first 10-ml. aliquot from each 50 ml. distilled was tested for purity by its effect on a standard cholesterol solution, human serum, and a protein and high-density lipoprotein fraction of human serum (*11*)—the infranate of serum separated at 1.063 gm. density in a preparative ultracentrifuge. Acetic acids from Baker and Adamson, J. A. Baker Chemical Company, Mallinckrodt Chemical Works, E. I. du Pont, and Merck and Company, Inc., have all been found to need distillation, but the quantity of impurity has been found to vary from batch to batch within each company's product. In the distillation experiment reported only one batch of acid was used. After proper distillation the acids from all sources are equivalent.

## Results and Discussion

The error in serum cholesterol values caused by the reaction of glyoxylic acid in undistilled acetic acid with tryptophan from human serum was investigated by following the course of acetic-acid distillations. A standard cholesterol solution, human serum, and the protein and high-density lipoprotein component (hereafter called the protein solution) of human serum were used in the test. The protein solution was selected for use because it contains all the serum proteins and the high-density lipoproteins, which contain only a small percentage of the total serum cholesterol. This would enhance any error caused by the reaction of the tryptophan from the protein with glyoxylic acid when enough of the material was used to insure a measurable amount of cholesterol. Five different types of distillations were made: (1) plain, with no modifications; (2) under an $N_2$ atmosphere; (3) from $FeSO_4$, 1 gm. per liter, under an $N_2$ atmosphere; (4) after 3 hr. refluxing in the presence of zinc granules under $N_2$; and (5) from a $K_2Cr_2O_7$ (0.1 gm. per liter) solution. Distillation 5 was from an oxidizing solution as first suggested by MacIntyre and Ralston (*4*) and confirmed by Morris (*2*) and Henly (*5*). The use of $K_2Cr_2O_7$ in an acid solution is equivalent to the use of $CrO_3$ reported by these investigators. Distillation Types 1–4 gave similar results, varying only a little in the time to reach plateau values. Figure 1 shows the typical result.

Distillation Type 5 differed. The plateau values shown in Fig. 1 were reached as soon as the boiling point of acetic acid was reached, and these values continued until the concentration of $K_2Cr_2O_7$ became so high (due to removal of acetic acid) that secondary oxidation began

to take place as shown by a rapid change of color and a rise in the temperature. Refluxing the acid prior to distillation is not necessary. All acid used for subsequent experiments was distilled from $K_2Cr_2O_7$.

The most striking finding from this experiment is that there are at

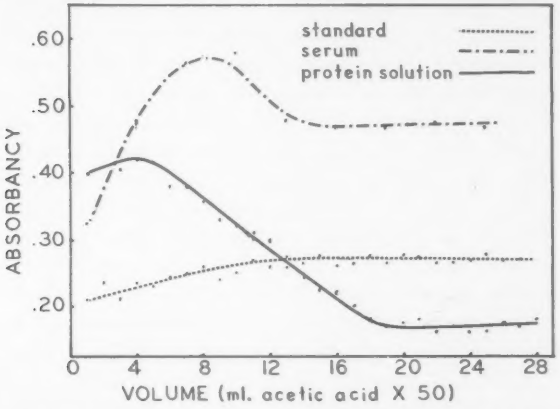


**Fig. 1.** Distillation of acetic acid from $FeSO_4$ under $N_2$ is typical of all distillations. Effect of impurities in commercial acetic acid on intensity of color developed in reaction of cholesterol with $FeCl_3$ color reagent.

least two different impurities in commercial acetic acid. This is shown by the rising values of the standard indicating one impurity, and by the decreasing values of the protein solution and the serum curves indicating an overriding second impurity. This second impurity could be the glyoxylic acid. The first impurity is hitherto unreported. It may be peracetic acid which could oxidize the cholesterol to cholestenone (*12*), thus reducing the amount of color formed by the color reagent, as cholestenone does not affect the $FeCl_3$ reagent (*13*). (This impurity has been found in most but not all acetic acid batches tested.) The presence of this impurity and the reducible substance glyoxylic acid led to the use of reducing agents prior to or during distillation. Reducing agents appear to improve distillation results slightly but not in a manner which is conclusive. Errors caused by the two impurities are large. The immediate removal of these impurities from acetic acid when distilled from $K_2Cr_2O_7$ make this the method of choice. The elimination of these two impurities make the direct serum cholesterol values much more comparable to values obtained through extraction

procedures. Specifically, when compared to the procedure of Abell *et al.* (*14*) using 21 consecutive human sera, the mean values were 316 ± mg./100 ml. for the $FeCl_3$ procedure and 310 ± 3.3 mg./100 ml. for the Abell procedure.

Furst and Lange (*8*) found that changes in the initial temperatures of reagents caused changes in the amount of color developed by the Zlatkis color reagent. There is an upward trend to absorbancies read with respect to an increase in temperatures of the Rosenthal reagents. Alterations in absorbance over a range of temperatures from 2° to 47° are well within the range of ± 3%, which is the range of reproducibility. Certainly the comparatively small variations in room temperature which would occur during the course of an analysis would cause errors so small as to be negligible, especially when compared to a standard done at the same temperature. If the procedure as described is followed carefully, heats of reaction will be uniform and error from this source negligible.

Furst and Lange also noted a day-to-day difference in color development. This is also true of the Rosenthal color reagent. This is demonstrated by a daily decrease in the amount of color developed by the cholesterol standard. The ferric chloride reagent itself becomes noticeably less yellow. This necessitates measuring a standard daily. In order to have a control, a single serum was pipetted fresh into suitable containers and frozen until used. Measurements were made on this control serum each time a new standard or a new color reagent solution was made up. Values obtained over a period of a few months from any control serum so used were reproducible within experimental error.

An effort was made to confirm the results of Kinley and Krause (*9*) concerning the error in cholesterol values caused by vitamin A. The study was expanded to include the oil-soluble vitamins D, E, and K, and also to include nicotinic acid. The results are shown in Table 1.

The vitamins measured had to be in milligram or gram quantities to obtain the small errors noted. If these concentrations are extrapolated to physiologic values, the error becomes immeasurably small. The report of Kinley and Krause stated that chloroform was used to dissolve the vitamin A added to the serum they measured. We have found that 0.025 ml. of chloroform added to a standard caused an increase of 11 mg./100 ml. in the cholesterol standard. It is also notable that in their report Kinley and Krause could not correct for physiologic vitamin A, but only for added vitamin A.

**Table 1.** Error in Cholesterol Values Caused by Added Vitamins

| *Solution* | *Added vitamins per 100 ml. (mg.)* | *Cholesterol value per 100 ml. (mg.)* | *Error caused by vitamins per 100 ml. (mg.)* | *Physiologic serum concentrations* |
|---|---|---|---|---|
| Cholesterol standard | 0 | 201 | 0 | — |
| Vitamin A | 15 | 208 | 7 | 22.4-78.0 μg. (*16*) |
| Vitamin D | 2.5 | 245 | 44 | 1.73-3.4 μg. (*17*) |
| Vitamin E | 400 | 219 | 18 | 1.00 mg. (*18, 19*) |
| Vitamin K | 50 | 201 | 0 | Not known |
| Nicotinic acid | 20 | 204 | 3 | 20 μg. (*19*) |

The error in cholesterol values due to hemolysis of the serum was investigated. A standardization solution of hemoglobin, made from hemolyzed washed red cells, was prepared, and portions of this solution were added to serum for measurement. The error is significant, but it is not proportional to the concentration (Table 2).

Sera, not plasma, should always be used for cholesterol determinations. Plasma from blood containing the anticoagulants oxalate, citrate, and fluoride gives values about 30–50 mg./100 ml. lower than sera. Plasma containing heparin or EDTA also give low values, but in the range 5–15 mg./100 ml. The use of any of these anticoagulants, in concentrations found in plasma, with a pure cholesterol standard does not cause an appreciable error. Values obtained on plasma and sera using the direct method were duplicated using the method of Abell *et al.* (*14*). The results indicate that serum should be used in that extraction procedure also. The anticoagulant effect is probably due to increased osmotic pressure which dilutes plasma with cell water.

A high concentration of chloride ion will also cause an error in cholesterol values. This error becomes appreciable in the high concen-

**Table 2.** Error in Serum Cholesterol Values Caused by Hemolysis

| *Solution* | *Hemoglobin (mg./100 ml.)* | *Cholesterol (mg./100 ml.)* | *Hemolysis error (mg./100 ml.)* | *Solution color* |
|---|---|---|---|---|
| 1 | 0 | 104 | 0 | Yellow |
| 2 | 1.875 | 110 | 6 | Yellow |
| 3 | 3.75 | 112 | 8 | Amber |
| 4 | 18.75 | 119 | 15 | Pink |
| 5 | 37.5 | 122 | 18 | Decidedly pink |
| 6 | 187.5 | 133 | 29 | Red |
| 7 | 375.0 | 148 | 44 | Very red |

trations used in ultracentrifugation. This error is discussed in full by Boyle *et al.* (11).

Bischoff and Turner (*13*) tested a number of steroids with the ferric chloride reagent and reported that most have no effect. They also noted that lathosterol, a cholesterol precursor, would cause some error with the ferric chloride reagent, but would cause a much greater error with the Liebermann-Burchard reagent. Desmosterol in patients treated with MER/29* appears to affect Liebermann-Burchard and ferric chloride color reagents similarly. Cholesterol values obtained in this laboratory by the two color reagents on persons treated with MER/29 are similar. This is in agreement with Frantz *et al.* (*15*).

Bilirubin will cause error, but that error in the great majority of sera would be negligible because of the low physiologic concentrations. In those sera which have a high bilirubin value, a correction can be applied by substracting 1.4 mg. of cholesterol per 100 ml. of serum for 1 mg. of bilirubin per 100 ml. of serum (*4*). These values have been confirmed in this laboratory.

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# A Simplified Method for Determination of Urinary Lead

*Russell Hilf* and Frank F. Castano*

**A modification for simplifying the quantitative determination of urinary lead by reaction with dithizone is described. The detailed procedure is presented and data on recoveries are given. The method takes less time than the original procedure with no loss in accuracy.**

THE INHALATION of lead and its salts, in the form of fumes or dust, has long been a problem in the industrial usage of lead. Awareness of this problem has led to several methods of determining lead exposure in suspected persons (*4*). One method which has become widely used is the determination of urinary lead levels.

A method which is most suitable was described by Cholak *et al.* (*2, 3*), and the procedure has been used with success in this laboratory. Because of the large number of samples received by our laboratory, modifications were undertaken to perform the assays in shorter time intervals. Contamination is a major problem and all glassware and equipment which comes in contact with the sample must be scrupulously cleaned prior to use. We therefore felt that elimination of as many transfers as possible would shorten the time for the determination, lessen the possibility of loss due to handling, as well as prevent any outside contamination. This report gives details of a modification which confines the entire procedure, up to the spectrophotometric reading, to the original specimen bottle.

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From the Chemistry Division, 6th U.S. Army Area Medical Laboratory, Fort Baker, Calif.

The authors wish to acknowledge the technical assistance of Pvt. Frank Siegel and of Major Robert L. Krivulka, for suggesting the use of wide-mouth, screw-cap bottles with polyethylene liners for submission of urine specimens.

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## Materials and Methods

### Reagents

All reagents are made up in deionized distilled water using glassware which has been rinsed twice with 8% nitric acid, 3 times with deionized distilled water, and oven dried.

1. *Precipitating solution* To 2.5 gm. of calcium carbonate, add just sufficient hydrochloric acid to effect solution. Then add 2 gm. of diammonium phosphate and make up to 100 ml. with deionized water. If phosphate precipitates, add hydrochloric acid drop by drop until solution clears.
2. *Ammonia water solution* 1% (W/V)
3. *Hydrochloric acid* 0.65% (W/V)
4. *Buffer solution* To 400 gm. of citric acid, add 280 ml. of deionized water. Add concentrated ammonia water until solution is just alkaline to phenol red. Cool, add 20 gm. of potassium cyanide and 10 gm. of sodium sulfite and dilute to 1 L. with deionized water. Store in refrigerator and make up fresh each month.
5. *Dithizone solution* For 24 samples (including one blank and two standards), place 12.1 mg. dithizone in 520 ml. of ammonia water (sp. gr., 0.90). Shake well and, within 15 min., filter the dithizone solution through cotton into 260 ml. of buffer solution. Stir periodically. This solution must be made fresh prior to each use.
6. *Stock lead standard* Dissolve 120 mg. lead nitrate in deionized water to make 1 L. of solution. This solution contains 75 mg. Pb per liter.
7. *Working standard* Solution contains 150 $\mu$g. of lead per liter. One milliliter of the stock solution is diluted to 500 ml. with deionized water to give the working standard.
8. *Chloroform* Reagent grade.

### Specimens

Urine specimens are submitted in screw-cap, wide-mouth, round, 130-ml. bottles. Caps are fitted with washable plastic liners. These bottles are washed 2–3 times with 8% nitric acid, followed by 2–3 rinses of deionized water, and dried in an oven. These bottles are supplied by the laboratory to hospitals and dispensaries requesting urinary lead determinations. The entire quantitative procedure is carried out in this specimen bottle. The patient is instructed to void directly into the bottle, taking care to avoid touching any part of the bottle or the cap, except the outside.

### Procedure

1. Measure out 50 ml. of deionized water into one bottle (blank).
2. Measure out 50 ml. of working lead standard into two different bottles (standard in duplicate).
3. To each bottle, add 1 ml. of lead precipitating reagent.
4. To each bottle, add 1 ml. of concentrated ammonia water (sp. gr., 0.90) and let stand 10 min.
5. Centrifuge for 5 min. at 2000 rpm. (Centrifuge used is an International, Size 2.)
6. After centrifugation, decant supernatant liquid into 100-ml. cylinders and record the volume. Discard supernatant.
7. To the precipitate add 25 ml. of 1% ammonia water solution and shake for a few seconds.
8. Centrifuge for 5 min. at 2000 rpm.
9. Decant and discard supernatant.
10. Add 25 ml. of 0.65% HCl and mix until precipitate dissolves.
11. Add 10 ml. of chloroform.
12. Add 30 ml. of buffered dithizone solution (freshly prepared). Shake well.
13. Centrifuge for 3 min. at 2000 rpm.
14. Draw off supernatant with suction and discard. Transfer chloroform layer to Coleman spectrophotometer cuvette (19 × 105 mm).
15. Read absorbance at 520 mμ, using Coleman spectrophotometer.
16. Calculate lead concentration using following formula:

$$\frac{\text{O.D. unknown}}{\text{O.D. standard}} \times \frac{7.5\ \mu\text{g.}}{\text{unknown vol.}} \times 1000 = \mu\text{g. Pb/L.}$$

The following pretreatment is used when the ingestion or exposure to organic lead compounds is suspected. The urine sample is first treated by the addition of 1.5 ml. of an acid solution of potassium iodide: iodine (150 mg. potassium iodide, acidify with nitric acid, 75 gm. iodine, and make up to 250 ml. with deleaded distilled water) for each 50 ml. of urine sample. The digestion is accomplished by heating at 80° for 20 min. After cooling, 1 ml. of sodium sulfite (20%) is added to reduce any of the unreacted iodine. The sample is then carried on as the procedure is outlined.

## Results and Discussion

The outlined procedure given above has been used for several years in this laboratory. The major advantage is the confining of the entire

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The following pretreatment is used when the ingestion or exposure to organic lead compounds is suspected. The urine sample is first treated by the addition of 1.5 ml. of an acid solution of potassium iodide: iodine (150 mg. potassium iodide, acidify with nitric acid, 75 gm. iodine, and make up to 250 ml. with deleaded distilled water) for each 50 ml. of urine sample. The digestion is accomplished by heating at 80° for 20 min. After cooling, 1 ml. of sodium sulfite (20%) is added to reduce any of the unreacted iodine. The sample is then carried on as the procedure is outlined.

## Results and Discussion

The outlined procedure given above has been used for several years in this laboratory. The major advantage is the confining of the entire

procedure to the original sample bottle. This eliminates the use of rather large numbers of separatory funnels, centrifuge tubes, and other glassware as recommended in the original procedure and prevents the loss of material in transfer. It does not eliminate contamination of the original urine sample, a problem which is beyond the control of the laboratory.

Figure 1 shows a typical standard curve. The method follows Beer's law from 30–150 μg. Pb per liter. Below 30 μg./L. of lead, no correlation between concentration and absorbance was obtained. However, this was not considered to be a serious objection since values below the concentration of 30 μg./L. would certainly fall within the accepted normal excretion.

Recovery experiments were performed employing normal urine samples and adding known quantities of inorganic lead. These results are recorded in Table 1. Good recoveries were obtained in the range

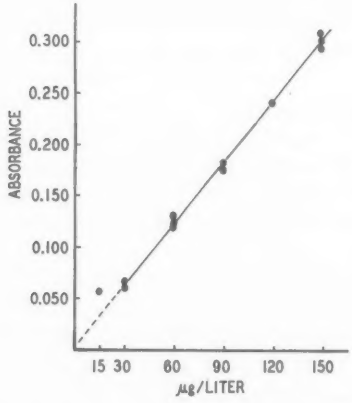


**Fig. 1.** Standard curve for colorimetric determination of lead.

**Table 1.** Recovery of Lead in Urine

| *Expected concentration (μg. Pb/L.)* | *Calculated concentration* (μg. Pb/L.)* | *% Recovery* |
|---|---|---|
| 15 | 22.8 | 152 |
| 30 | 49.1 | 164 |
| 60 | 60.4 | 101 |
| 90 | 103 | 115 |
| 120 | 112 | 93 |
| 150 | 142 | 95 |

*Figures represent the mean of three different determinations for each level of lead added to urine.

of 60–150 μg. Pb per liter. It has been a practice of this laboratory to report the exact lead concentration of any sample containing 50 μg./L. or more. Because of the discrepancy in the literature as to the normal urinary lead content, we have assumed that any urine containing more than 50 μg. lead per liter is worthy of notation and suggest additional samples be submitted for confirmation (*1*).

With the above procedure, we have found it quite easy to perform the analysis of 21 unknown samples plus the one blank and two standards in a relatively short period of time. This has greatly simplified the method and resulted in a substantial saving of time, one person being able to run 20–40 samples per day.

## Summary

A modification of the urinary lead procedure is presented. The entire analytical method is confined to the original sample bottle, thus eliminating excessive glassware and possible outside contamination and providing a substantial saving of time. Recovery studies indicated no loss of accuracy.

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# A Rapid and Sensitive Turbidimetric Method for Serum Lipase Based Upon Differences Between the Lipases of Normal and Pancreatitis Serum

*William C. Vogel and Leslie Zieve*

**A rapid turbidimetric method for the determination of serum lipase is described. The substrate is a nearly pure triglyceride in Tris buffer, with sodium deoxycholate as the emulsifying agent. The method is equal in sensitivity to the titrimetric measure of serum lipase using a modified Cherry-Crandall substrate.**

**The serum of patients with acute pancreatitis contains a lipase with optimal action at *p*H 9.1 and sodium deoxycholate concentration of 0.35%. These are different from the optimal *p*H and bile salt requirement for lipase activity in normal serum or duodenal contents.**

In a previous study (*1*) we presented data suggesting that the measurement of serum lipase is of greater value than serum amylase in the diagnosis of pancreatitis. For that study the modified Cherry-Crandall substrate of Tietz and co-workers (*2*) was used. The preferred use of olive oil rather than other substrates in the detection of "pancreatitis lipase" has been reviewed by Henry (*3*), who emphasized that the lipase in the serum of patients with pancreatitis has properties differing from the lipase in pancreatic extracts. The titrimetric method, however, has a limited application in the clinical laboratory due to the time required for an analysis.

Our attention was directed to the use of turbidimetric measurements of lipase action which are now often preferred to titrimetric assays (*4*). The rate of clearing of the turbidity of a triglyceride emul-

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sion as a measure of lipase action has been presented most recently in the methods of Guth and co-workers (*5–7*) and Borgstrom (*8, 9*).

While the methods of these workers, as described, did establish the elevation of serum lipase in clinically apparent acute pancreatitis, they were less effective, in our hands, than the titrimetric method in distinguishing the patients with mild or convalescent pancreatitis. The rapid turbidimetric modification presented in this report is equal in sensitivity to the usual titrimetric assay. With careful preparation of the substrate, the method is readily applied in the routine clinical laboratory.

## Method

### Reagents

*0.05 Molar Tris buffer pH 9.1:* 6.057 gm. of Fisher Cat. No. T-395 Tris (hydroxy-methyl) aminomethane is dissolved in approximately 980 ml. of water and adjusted with 1N HCl to a *p*H of 9.1 at a volume of 1 L.

*Buffer diluent (with 0.35% deoxycholate):* 3.50 gm. of Difco Laboratories Cat. No. 0248-13 sodium deoxycholate is dissolved in and diluted to a volume of 1 L. with the above buffer. Readjust to *p*H 9.1 if necessary.

*Purified Olive Oil:* Fisher Cat. No. 0-111, Best, U.S.P. Olive Oil is passed through alumina to remove free fatty acids. We have used Merck Cat. No. 71707 reagent aluminum oxide, suitable for chromatographic adsorption, and a 2 × 32 cm. chromatographic tube fitted with a coarse fritted glass disk. For a column height of 8 cm. of alumina, pour in olive oil to approximately double this amount. After 1½–2 hr., the nearly colorless effluent olive oil is collected.

*Stock olive oil solution:* 10.0 gm. of the treated olive oil is dissolved in reagent grade acetone to a volume of 100 ml.

*1% Olive oil solution:* 10.0 ml. of the stock solution is diluted to a volume of 100 ml. with acetone.

### Equipment

Waring Blendor with the semimicro (350-ml. capacity) Monel metal homogenizer; a variable voltage transformer; Coleman Junior Spectrophotometer Model 6A with adapter to use cuvettes 75 × 12 mm O.D.; and a water bath at 38°.

### Preparation of the 0.04% Triglyceride Substrate

The metal homogenizer containing 100 ml. of the buffer diluent is set in a vigorously boiling steam-heated water bath. When the temper-

ature of the diluent reaches 92° (approximately 4 min.) the homogenizer is removed from the bath and placed on its base which is connected to the transformer set at 35% of output. This slower speed is used to avoid froth formation during the addition of olive oil. Then 4.0 ml. of the 1% olive oil solution is added at once, dropwise, over a 2-min. interval. The homogenizer is then run uncovered at full speed for 10 min. The intense froth which has formed will break completely within 5 min. The final volume of substrate of approximately 90 ml. is transferred to a stoppered flask and cooled to room temperature. Acetone is expelled in the process of preparation. This substrate may be used the day of preparation; however it cannot be considered stable. If used more than 2 hr. after preparation, it should be placed on a mechanical mixer for 5–10 min. because a fine sediment appears on the bottom of the container. The loss in sensitivity up to 3 days after preparation is shown in Table 1. Also the variation in lipase attributable to substrate variability is indicated by the values in the first three and the last columns.

With pigmented serums, the initial optical density of the incubation

**Table 1.** Effect of Age of Substrate on Turbidimetric Lipase Values (Units)

| | *Original analysis Using three separate substrates* | | | *Repeat analysis Using three separate substrates* | | | *Substrate used 8 hr. after preparation* |
|---|---|---|---|---|---|---|---|
| *Serum* | *No. 1* | *No. 2* | *No. 3* | *No. 3 + 1 day* | *No. 2 + 2 days* | *No. 1 + 3 days* | |
| 1 | 236 | 226 | 222 | 196 | 221 | 184 | 241 |
| 2 | 72 | 71 | 78 | 59 | 67 | 55 | 76 |
| 3 | 43 | 40 | 49 | 37 | 41 | 31 | 47 |
| 4 | 6 | 8 | 5 | 2 | 8 | 6 | 6 |
| 5 | 25 | 28 | 22 | 17 | 18 | 16 | 28 |
| 6 | 22 | 21 | 24 | 12 | 17 | 17 | 34 |
| 7 | 29 | 30 | 27 | 12 | 24 | 19 | 27 |
| 8 | 4 | 5 | 6 | 1 | 0 | 3 | 3 |
| 9 | 0 | 4 | 6 | −2 | 1 | −4 | −3 |
| Substrate absorbance | 0.526 | 0.504 | 0.521 | 0.509 | 0.521 | 0.519 | 0.491 |

Serum turbidimetric lipase values were determined for 3 successive days with a fresh substrate used within 2 hr. of the time of preparation. On the fourth day these substrates, which had been kept at room temperature, were remixed for 10 min. on a mechanical mixer and the serum lipase again determined. In addition, on the fourth day a fresh substrate was prepared and after 8 hr. was mechanically mixed before analysis.

Serums 1–4 were obtained from a case of acute pancreatitis on the first, second, seventh, and twenty-eighth days of the illness. Serums 5–7 were obtained on the first, second, and fourth days from a case of mild pancreatitis. Serums 8 and 9 were from healthy adults. Serums were frozen between intervals of analysis.

mixture may exceed 0.700, and for this reason the triglyceride concentration of 0.04% was not exceeded. The described manner of preparing the substrate gives a nearly reproducible substrate absorbance. While substrates with larger particle size (lower absorbance) may show an equivalent initial reaction, the rate is not as uniformly sustained as with the preparation employed.

### Procedure

Only serum has been used in this study. It must be diluted 1:5 with the buffer diluent. Then 4.0 ml. of the substrate is placed in the cuvette and equilibrated in the 38° water bath. The spectrophotometer is adjusted to zero absorbance at 400 m$\mu$ with a cuvette containing the buffer diluent; 0.2 ml. of the diluted serum is added to the substrate cuvette, mixed by inversion 5 times against a cap of parafilm and the zero time absorbance recorded. The absorbance is read again after 20 min. at 38°. The difference between the zero time and 20-min. absorbance reading $\times 10^3$ is recorded as the result. Thus 1 lipase unit is a change in absorbance of 0.001 under the conditions specified.

A few experiments have shown that EDTA (ethylenediaminetetraacetate) is inhibitory to lipase of pancreatic origin; hence all calcium-binding anticoagulants should be avoided until they are demonstrated to have no effect in this system. Unhemolyzed serum is desirable. However, in our limited experience with cases of convalescing pancreatitis, an occasional hemolyzed serum has not distorted the sequence of progressively decreasing lipase values shown by the unhemolyzed serums. We have not specifically studied the effects of hemolysis.

### Comparison with Titrimetric Method

While change in turbidity of a triglyceride emulsion may be correlated with a reaction product of lipase action, we have elected to compare the change in absorbance in the method proposed, to the increase of fatty acids as measured in a titrimetric method using the modified Cherry-Crandall substrate of Tietz and co-workers (*2*) at a *p*H of 8.0. In this titrimetric measure of fatty acids released, we have applied the microtechnic of Dole (*10*) which permits serial determinations of 1.0-ml. samples during the incubation. As previously reported (*1*), the $\mu$Eq. of fatty acids released during a 3-hr. incubation are recorded as units of lipase action. A zero time sample after the addition of the serum is subtracted from the 3-hr. 1.0-ml. titration

value, to correct for the variation in fatty-acid content of serum. It is assumed that the increase of fatty acids during the incubation is due only to lipase action.

The correlation of the turbidimetric and titrimetric methods is presented in Fig. 1. Taking 15 U. as a conservative upper limit of normal for the turbidimetric method and 30 U. (*1*) for the titrimetric method, the two procedures are approximately equal in sensitivity in the region of mild abnormality. All subsequent normal serums have fallen

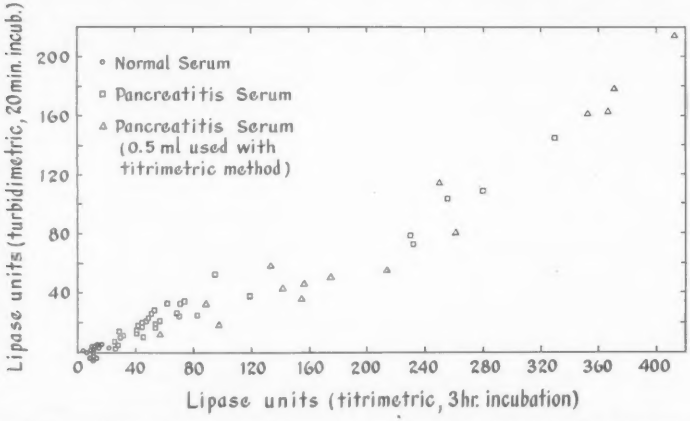


**Fig. 1.** Correlation of serum lipase determined with turbidimetric and titrimetric methods. Serum lipase concentration was determined by two methods described in text in 15 healthy adults and at one or more intervals in 17 cases of acute and convalescent pancreatitis. Values based upon 0.5 ml. of serum are plotted after multiplying by two. Resultant error is described in text and is shown in Fig. 2.

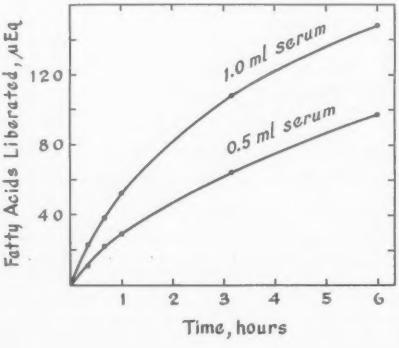


**Fig. 2.** Release of fatty acids in a modified Cherry–Crandall substrate. Release of fatty acids at intervals shown was measured in substrate and by technic described in text. Same serum, a pool of serum from patients with mild acute pancreatitis, was used for both curves. With 0.5-ml. sample, extra 0.5 ml. of water was added to substrate before addition of serum and determination of zero-time fatty-acid content.

in the range of +5 to −5 U., with the exception of one value of 7 and one of 11 U. Values of +5 to −5 may be observed in controls of substrate plus diluent. The need for the emphasis in Fig. 1 that certain serums were analyzed with 0.5 ml. serum is demonstrated in Fig. 2. It has been emphasized previously (*3*) that neither the proportion of serum to substrate nor the length of time of incubation can be altered in the titrimetric test. It can also be emphasized from Fig. 2 that another serum with twice as much pancreatitis lipase per milliliter would not liberate twice as much fatty acid. It will be shown that this limitation in measuring the degree of severity of pancreatitis is also inherent in a turbidimetric method in which readings are taken at a definite time interval.

## Experimental Studies

### Purity of Substrates

A modification of the existing turbidimetric methods was necessary when neither of these showed an elevation in serum lipase in mild pancreatitis. The development of the present method was dependent upon the use of a nearly pure triglyceride with a natural emulsifying agent to prepare an emulsion of reasonably reproducible absorbance at a *p*H of 9.1.

Through the use of chromatography of nonphosphatides as described by Marinetti and Stotz (*11*), and the rapid thin-layer silicic acid technic (*12*), it was found that the expensive commercial triolein that is available may contain numerous other components. Thus one triolein showed six components with the thin-layer technic. The passage of inexpensive olive oil through alumina yields a triglyceride free of fatty acids with a marked reduction in the amount of two other components. This is shown in the photograph of a thin-layer chromatogram in Fig. 3, which also shows the mobility of other lipids. The pure triglyceride of olive oil may be obtained after the alumina treatment by further elution from silicic acid with Solvent 1 of Papariello *et al.* (*13*).

The use of alumina-treated or untreated olive oil in the substrate was compared with the following results. Normal serums are the same with near zero action. Five serums with lipase values from 21 to 52 U. gave values with untreated olive oil from 4 to 42 U., averaging 61% of treated olive oil values. Of five serums with lipase values from 51 to 214 U., two were the same with untreated olive oil, while the other three averaged 65% of the values obtained with the treated olive oil.

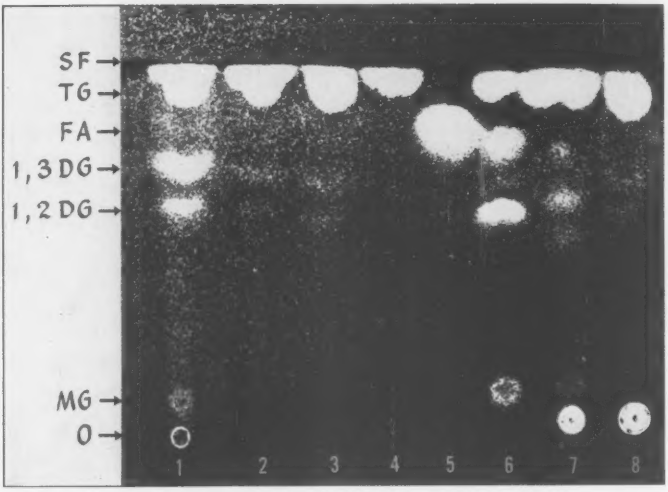


**Fig. 3.** Photograph of a thin-layer silicic acid chromatogram. Two reagent-grade triolein samples were applied at *1* and *2*, olive oil before and after alumina treatment at *3* and *4*, reagent-grade oleic acid at *5*, an ethyl ether extract of an acidified partial digest of the Tietz substrate with the lipase of duodenal aspirate at *6*, Ediol at *7*, and Lipomul at *8*; 40-μl. aliquots of 2% (v/v) solution of lipids in chloroform: methanol (2:1 v/v) were applied at respective origins with exception that 20 μl. was applied at *5*, and a suitable aliquot at *6*. Abbreviations: SF, solvent front; TG, triglyceride; FA, fatty acids; DG, diglyceride; MG, monoglyceride; and O, origin.

### Effect of Heating Substrate

The thin-layer chromatographic technic was used to determine whether any alteration of triglyceride occurred during the heat of substrate preparation. Comparison was made with a substrate prepared by adding the acetone solution of triglyceride slowly, with mixing, to cold buffer diluent, followed by vacuum removal of the acetone (*9*). Apparently a complex is formed in the heated substrate. Initial chromatograms of ethyl-ether extracts of heated substrates acidified with acetic acid showed a streak from the origin to the solvent front. However, when more strongly acidified, a single spot of triglyceride was obtained comparable to that shown by the extract of the substrate prepared in cold diluent.

### Effect of Bile Salt

It has been noted (*8*) that synthetic emulsifiers may be competitive inhibitors of lipase. Sodium deoxycholate used here was found most

effective at nearly the same concentration found optimum for duodenal lecithinase A action on lecithin in an aqueous system (*14*). The initial selection of 0.35% deoxycholate was based on a sharp optimum at this concentration shown by serum from 1 severe case of pancreatitis, using an impure triolein (*1* in Fig. 3) as substrate. Additional studies (Fig. 4) with alumina-treated olive oil as substrate show this to be a desirable concentration for detecting pancreatitis lipase in serum. The pool of pancreatitis serums utilized included some serums that were nearly normal in lipase content. Apparently there is a lower deoxycholate requirement for optimal action of normal serum lipase.

The usefulness of sodium deoxycholate in the measurement of human serum lipase may not apply to the lipase of other species. Borgstrom (*8*) found that taurocholic and cholic acid increased the action

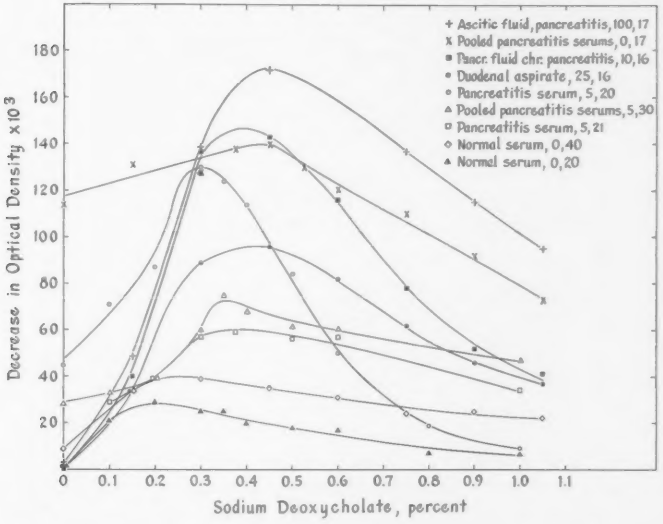


**Fig. 4.** Variation in optimal sodium deoxycholate concentration at *p*H 9.1. Emulsified triglyceride was prepared as described in text except that Tris buffer without deoxycholate was used. Immediately after 10-min. interval on homogenizer, aliquots were transferred to tubes containing varying amounts of dry sodium deoxycholate, to yield concentrations shown in figure. Separate substrates showed only slight variation in optical density or *p*H.

Lipase action was measured as described in text. Extent of dilution of enzyme source in buffer diluent, and variation in time (minutes) of reading of optical density change, are given, respectively, by two numbers following legend corresponding to each symbol. All readings employed reference of buffer alone.

of rat pancreatic lipase at an acid *p*H, but inhibited the lipase in an alkaline substrate.

### Effect of *p*H

Although 0.35% sodium deoxycholate is not optimal for all sources of lipase shown in Fig. 4, this concentration was used in a study of the effect of *p*H on lipase action (Fig. 5). It is evident that proper selection of the *p*H of the substrate will increase sensitivity to pancreatitis lipase while minimizing the action of normal serum lipase. *p*H 9.1 is well above the optimal *p*H for lipase of normal serum and approximates the peak of action for lipase of pancreatitis serums studied with this technic. Two lipase components of normal serum are indicated,

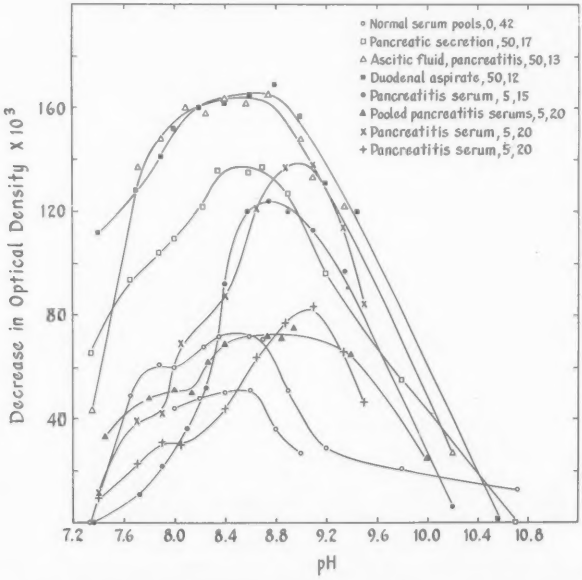


**Fig. 5.** Variation in optimal *p*H at sodium deoxycholate concentration of 0.35%. Substrate was prepared as described in text. Then 10.0-ml. aliquots were added to 0.5-ml. amounts of water, dilute acid, or alkali. Resultant *p*H was measured with a Beckman Zeromatic *p*H meter. There was but slight variation in substrate absorbance. Change of absorbance was measured with buffer diluent, *p*H 9.1, as reference. Dilutions of enzyme sources were made with this buffer diluent. 0.2-ml. samples were used with 4.0 ml. of substrate to measure change in absorbance. Dilution of enzyme source and interval of incubation are shown, respectively, by two figures after each enzyme source in legend.

one of which is near the optimal *p*H of pancreatic lipase. In acute pancreatitis a component with optimal action near *p*H 9.1 indicates that another or altered lipase has appeared. The possibility of the absence of an inhibitor or the presence of an activator is not dismissed. With one exception, the pancreatitis serums show the presence of the normal serum component(s). The curve for the pool of pancreatitis serums reflects its mixture of borderline and severe pancreatitis serums. The pancreatic secretion was from a patient with chronic pancreatitis and cannot be considered a true representation of normal pancreatic fluid. The extent of dilution of the nonserum sources may obscure the detection of minor lipase components.

### Effect of Wavelength and Temperature

Our choice of 400-m$\mu$ wavelength with the Coleman Jr. spectrophotometer was based on the fact that at this wavelength the greatest absorbance of the substrate was observed, and with clearing a greater change in reading would be obtained with slight lipase action (Fig. 6A). A comparison with readings obtained on the same reaction mixture at room temperature in the Beckman spectrophotometer set at 650 m$\mu$ is shown in Fig. 6B. Lipase action in serum dilutions of 1:10 or higher (effective incubation mixture dilutions of 1:210 or higher) are relatively depressed in Fig. 6A. It is shown in Fig. 6C and D with a different serum, that the depression of lipase action in dilute solutions of serum is related to the temperature of incubation. The change in absorbance at both 38° and 25° was measured with the Coleman instrument. For those who wish to use a Beckman spectrophotometer at room temperature, a correlation of readings of normal and pancreatitis serums at room temperature with the Beckman and at 38° with the Coleman is presented in Fig. 7.

In a triglyceride emulsion undergoing the action of lipase, the rate of removal of triglyceride will decrease, for as fatty acids are individually removed from triglyceride the enzyme may also act on di- and monoglyceride in the presence of the inhibitory effect of fatty acids. Under the conditions described by Borgstrom, an inverse relationship exists where 1 U. of enzyme takes twice as long as 2 U. to produce the same decrease in turbidity. One serum in a case of severe pancreatitis showed such a relationship, but the usual correlation is as presented in Fig. 6. This may be related to the variable composition of lipase components of pancreatitis serum, with varying optimal requirements of *p*H and deoxycholate concentration.

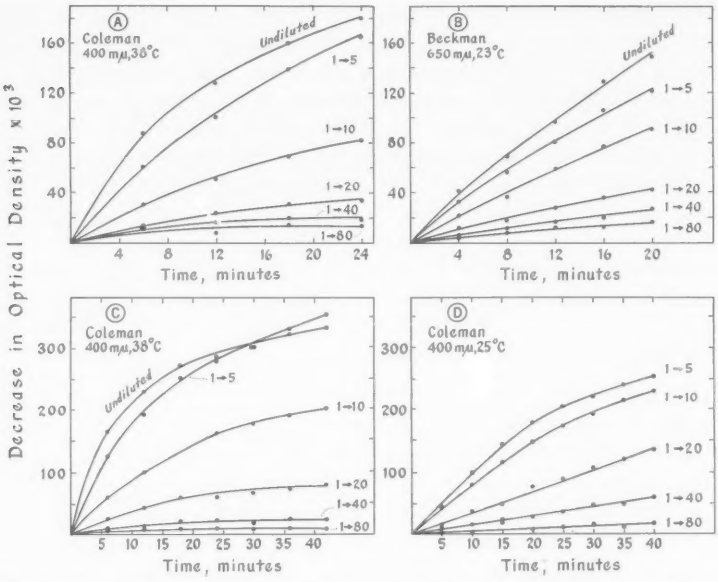


**Fig. 6.** Change in absorbance with variations in dilution, temperature, time, and instrument. In **A** and **B** change of absorbance of substrate is plotted with different dilutions of serum from case of pancreatitis, as measured in two spectrophotometers at wavelengths and temperatures shown. In **C** and **D** serum from another case of pancreatitis was used, and changes in absorbance at 38° and 25° were determined with Coleman instrument. In **B** substrate and serum sample were mixed in stoppered tube before transferring to 1-cm. cell for absorbance readings. Procedure for **A, C,** and **D** was as described in text.

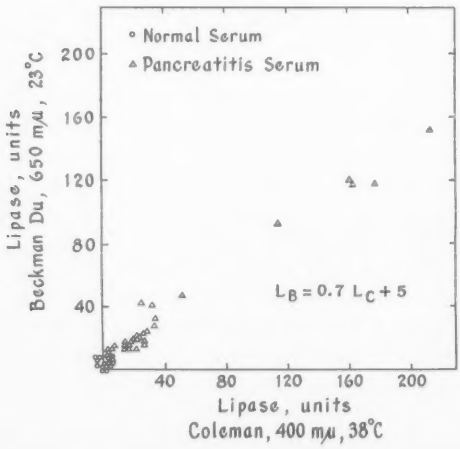


**Fig. 7.** Correlation of 20-min. change in absorbance as measured by two methods. Lipase action of serums from healthy adults and from 2 cases of pancreatitis was measured for a 20-min. interval with variation in instrument, temperature, and wavelength as shown. Procedure was as described in text, except that with Beckman instrument substrate and serum sample were mixed before transferring to 1-cm. cell for readings.

### Effect of Serum Dilutions

The 20-min. reading of change in turbidity of a 0.04% triglyceride emulsion will detect slight increases in lipase of serum, but does not reflect the true proportional increase in pancreatitis. The time required for a change of absorbance of even 0.050 would be an inconveniently long interval for mild pancreatitis, and too short an interval for accurate measurement in severe pancreatitis. A solution for those wishing to measure the true severity of the pancreatitis would be to record the length of time it takes for a change of O.D. of, say, 0.150. While the highest amylase in the serums of Fig. 1 was 1300 U./100 ml., we recently secured a pancreatitis serum with amylase concentration of 4600 U./100 ml. The change in turbidity of this serum at various dilutions is shown in Fig. 8. Also shown in the graph is the approxi-

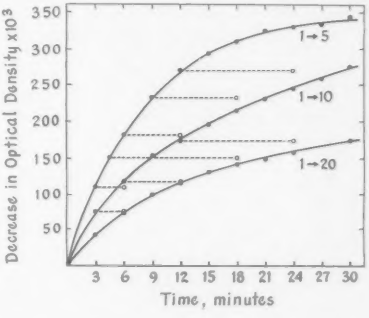


**Fig. 8.** Rate of decrease in absorbance with varying serum dilutions. Successive dilutions of serum from case of pancreatitis with amylase concentration of 4600 U./100 ml. were made with buffer diluent. Change of absorbance at various time intervals with mixtures of diluted serum and substrate was measured as described in text. Deviation from proportional relationship between reaction time and dilution is indicated by differences between curves and points at ends of horizontal lines.

mate proportionality of reaction time and dilution. The titrimetric method of Fig. 2 also exhibits an approximate proportionality between reaction time and dilution.

The 1:5 dilution of serum, through factors of temperature, *p*H, and deoxycholate concentration, effectively eliminates normal serum lipase action. Further separation of borderline pancreatitis from normal serum lipase cannot be accomplished by using undiluted serums. Five normal serums, with lipase values of 11, 3, −3, 1, and 7 U., were analyzed undiluted with the resulting values of 24, 18, 16, 24, and 32 U. respectively. Six borderline pancreatitis serums with values of 32, 27, 21, 28, 33, and 14 U., gave values of 29, 35, 49, 58, 73, and 38 U., respectively, when analyzed undiluted.

We have found that lipase values of serum are the same if the 1:5 dilutions are made with the Tris buffer alone. However, in initial stud-

ies with duodenal aspirations a loss of lipase action occurs with time when dilutions are made with the tris buffer. This loss can be prevented by using the buffer diluent. A period of contact with the deoxycholate of the diluent may actually slightly increase subsequent lipase action.

### Comparison with Synthetic Emulsions

Ediol* and Lipomul† were diluted to 1% triglyceride content with the Tris buffer. Then 2.0 ml. of each were mixed with separate 50-ml. amounts of the cold buffer diluent to form approximately 0.04% triglyceride substrates. The lipase units obtained with these substrates are compared to the one proposed in Fig. 9. The olive-oil substrate is clearly preferable. The differences in action may be due to the presence of synthetic emulsifiers or other lipid material (Fig. 3).

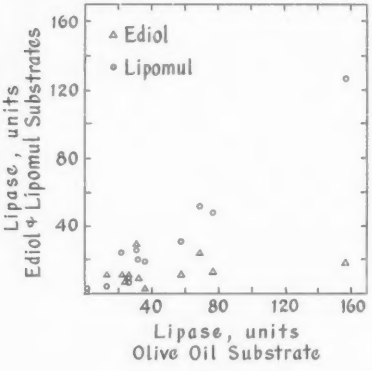


**Fig. 9.** Comparison of olive oil and synthetic emulsions as substrates. Substrates of Ediol and Lipomul were prepared as described in text. These were used in manner described for purified olive oil substrate. One normal serum and 10 serums from pancreatitis patients were diluted 1:5 with buffer diluent; an ascitic fluid from case of pancreatitis was diluted 1:100. Changes in optical density with three substrates were determined for a 20-min. interval and values for Ediol and Lipomul are plotted against those of purified olive oil as reference.

## Summary

1. The preparation of an emulsion of a nearly pure triglyceride of olive oil in Tris buffer, *p*H 9.1, and 0.35% sodium deoxycholate concentration, is described. This emulsion is utilized in a rapid turbidimetric measure of the lipase present in serum of patients with acute pancreatitis.

2. The method is shown to be as sensitive a measure of serum lipase as a titrimetric method which utilizes a modified Cherry–Crandall

*Schenlabs Pharmaceuticals, Northridge, Calif.
†The Upjohn Company, Kalamazoo, Mich.

substrate. The limitation in the measurement of high levels of lipase activity with either method is pointed out.

3. The serum of patients with acute pancreatitis contains a lipase which has an optimal *p*H and deoxycholate requirement which is different from the corresponding optima for lipase activity in normal serum or duodenal contents.

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# Clot Retraction and Fibrinolysis

*Murray Weiner*

**Three different systems were employed for parallel observations of clot lysis and retraction. Potassium and calcium concentration was found to influence significantly these phenomena in some systems but not in others. Under a variety of conditions, both retraction and fibrinolysis are affected in a parallel manner, suggesting that the retraction state of a clot used as a substrate may influence the apparent fibrinolytic activity of an enzyme system.**

PROTEOLYTIC ENZYMES are generally characterized by the nature of the substrates against which they are effective. Changes in the physical-chemical state of a given protein substrate may significantly alter the kinetics of its digestion by an enzyme. Some technics for studying fibrinolytic activity employ unretracted fibrin clots as substrate, while other methods use well-retracted clots. In some systems clot retraction is not readily distinguished from incomplete lysis.

This report summarizes some parallel observations of clot retraction and lysis. It is suggested that these two factors are not totally independent of each other.

## Methods

### Fearnley Technic (*1*) : Fibrinolytic Activation of Citrated Blood or Plasma by Dilution

Blood specimens were obtained from human subjects who had no evidence of coagulation disturbances or acute illness. Nine parts of blood were added to one part 3.8% sodium citrate. Retraction and lysis were observed in a water bath at 37° in glass tubes containing blood or plasma diluted 1:10 in sodium phosphate buffer (.07 M; *p*H,

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7.4), potassium phosphate buffer (.07 M; *p*H, 7.4), sodium chloride, 0.9%, or potassium chloride, 0.9%, and clotted with thrombin.

### Fibrinolytic Activation of "Undiluted" Plasma by Extrinsic Enzymes

To distinguish retraction from incomplete lysis, carmine, an inert, insoluble dye is included in the system as described below. With lysis, the dye is released and settles to the bottom of the tube. The color of the undissolved portion of the clot is not altered if lysis occurs without retraction. In contrast, retraction concentrates the dye within the clot, and no precipitate of dye is noted at the bottom of the tube (*2*).

To 0.2 ml. plasma, 0.05 ml. of a fibrinolytic agent,* 0.05 ml. of a suspension of carmine, and 0.1 ml. of water or the fibrinolytic inhibitor, $\epsilon$-aminocaproic acid, in varying concentration are added. The system is then clotted with 0.1 ml. $CaCl_2$ of varying molarity (0.025 to 0.5 M). The resultant fibrinolytic activity varies widely with each plasma specimen, presumably because of wide individual differences in inhibitor and antistreptokinase titer (*3*).

### Lysis of Thrombin-clotted Fibrinogen

Citrate-free lyophilized bovine fibrinogen (Chilcott) is reconstituted with water so as to contain 6.0 mg. clottable protein per milliliter. To 0.2 ml. of this solution, 0.01 ml. of the fibrinolytic preparation, 0.05 ml. saline or an $\epsilon$-aminocaproic acid solution, and 0.05 ml. carmine dye suspension are added. The system is then clotted with 0.05 ml. thrombin (Parke, Davis, 100 $\mu$g./ml.).

## Results

### Fearnley Technic

Twenty human citrated blood and/or plasma specimens were observed for retraction and lysis following dilution in both sodium and potassium phosphate buffer. In some instances plasma specimens which lysed readily in potassium phosphate buffer diluent failed to lyse at all in sodium phosphate buffer. The reverse has not been observed. Similarly, potassium chloride as a diluent was more likely to result in lysis than sodium chloride. Occasionally, whole blood sodium phosphate systems lysed, while the corresponding plasma sodium phosphate system failed to lyse. The reverse phenomenon was not observed in any instance. Apparently, the presence of potassium in this system, either from the diluent or possibly from red cells, may enhance fibrinolytic activity.

*Actase (Ortho), 2000 "units" per milliliter.

Retraction of sodium phosphate-diluted plasma clots was frequently poor compared to parallel potassium phosphate-diluted systems. These poorly retracted sodium systems often failed to lyse over a period of days. In contrast, the corresponding well-retracted potas-

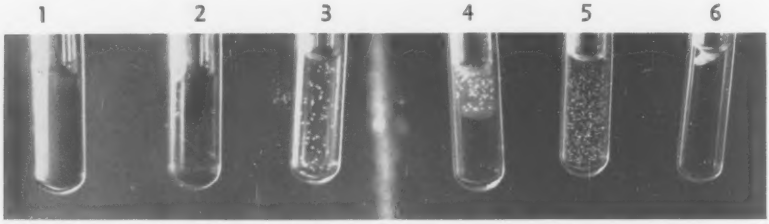


**Fig. 1.** Effect of incubation for half an hour of thrombin-clotted 1:10 dilutions of samples from the same specimen of citrated blood collected in a chilled silicone syringe and treated in the cold until diluted as follows:

1. Whole blood in Sodium Phosphate buffer
2. Whole blood in Potassium Phosphate buffer
3. Plasma in Sodium Phosphate buffer
4. Plasma in Potassium Phosphate buffer
5. Platelet-rich plasma in Sodium Phosphate buffer
6. Platelet-rich plasma in Potassium Phosphate buffer

sium phosphate diluted aliquot usually lysed completely in less than 24 hr.

The shrunken, more dense (i.e., retracted) clot frequently observed with potassium as compared to sodium phosphate diluent is illustrated in Fig. 1. This difference is noted with both whole blood or plasma, whether or not cold precautions are taken in handling the specimen prior to phosphate dilution. Tube 6 in Fig. 1 illustrates a more strongly retracted, platelet-rich specimen in potassium phosphate buffer. It lysed more rapidly than its corresponding ordinary plasma or whole-blood system. In this typical experiment the specimens diluted in sodium phosphate buffer (Tubes 1, 3, 5) all failed to lyse after 3 days.

Fearnley (*1*) has found it necessary to clot phosphate-diluted blood specimens with thrombin rather than calcium in order to obtain lysis, suggesting that in the phosphate system calcium inhibits lysis. This observation was readily confirmed. It was also found that in this phosphate-diluted system the retraction of the clot formed by adding thrombin is faster and more complete than that resulting from the addition of calcium.

### Whole Plasma: Fibrinolytic Agent System

Undiluted citrated plasma clotted by adding thrombin is known to result in a less firm and poorly retracted clot than that formed by

recalcification with $CaCl_2$—approximately 0.01 M in the final mixture (*4*). In the "undiluted" plasma system with added fibrinolytic reagent a 20-fold range of concentration of added $CaCl_2$ (0.025–0.5 M) did not influence rate of lysis (Table 1). However, the higher calcium concentrations had a marked inhibiting effect on clot retraction.

**Table 1.** Effect of ε-Aminocaproic Acid (EACA) and Calcium Concentration on Fibrinolysis and Clot Retraction in the "Udiluted" Plasma System*

| *EACA μg./ml. plasma* | *Clotted with .025 M $CaCl_2$* | | | *Clotted with 0.5 M $CaCl_2$* | | |
|---|---|---|---|---|---|---|
| | *10 min.* | *30 min.* | *24 hrs.* | *10 min.* | *30 min.* | *24 hrs.* |
| 0 | ++ | L | L | 0 | L | L |
| 0.25 | ++ | L | L | 0 | L | L |
| 2.5 | ++ | L | L | 0 | L | L |
| 25.0 | ++ | ++ | ++ | 0 | 0 | 0 |
| 250. | 0 | 0 | + | 0 | 0 | 0 |

*0 = no retraction or lysis.
\+ = incomplete retraction.
++ = strong retraction.
L = complete lysis.

In this system the well-recognized inhibitory effect of ε-aminocaproic acid on fibrinolysis was readily demonstrated (Table 1). In high concentrations (250 μg./ml. plasma), ε-aminocaproic acid also inhibited clot retraction.

### "Purified" Fibrin System

In the thrombin-clotted fibrinogen technic employed, the clot regularly retracted well and lysed within a few hours. Addition of ε-aminocaproic acid in final concentrations of 130 μg./ml. completely blocked fibrinolysis, and in lesser concentrations slowed fibrinolysis considerably. While there was no discernible effect from 130 μg./ml. ε-aminocaproic acid on clot retraction, concentrations of 1.3 mg./ml. completely inhibited retraction (Table 2).

**Table 2.** Effect of ε-Aminocaproic Acid (EACA) on Fibrinolysis and Clot Retraction in a Fibrinogen-Fibrinolysin System Clotted with Thrombin*

| *EACA mg./ml.* | *1 Hour* | *24 Hours* |
|---|---|---|
| 0 | L | L |
| 0.13 | 0 | ++ |
| 1.3 | 0 | 0 |

*0 = no retraction or lysis.
\+ = incomplete retraction.
++ = strong retraction.
L = complete lysis.

## Discussion

Unaltered normal clotted blood or recalcified citrated plasma does not lyse spontaneously in the test tube over a period of several days. Special technics are necessary to estimate the fibrinolytic potential of a given specimen. Some technics of observing lysis will detect lysis only in abnormal specimens or if lytic activity is artificially induced (*5*). Other methods (*3*) induce lysis of normal specimens by the addition of activator enzymes or by dilution with an appropriate nonenzymatic diluent. The possibility that alteration in the substrate fibrin may influence its susceptibility to lysis is frequently ignored. The data presented demonstrate that some factors (potassium, calcium, and platelets in dilution-activated systems; ε-aminocaproic acid in enzyme-activated systems) influence retraction and lysis in a parallel manner, while other factors (calcium concentration in an enzyme-activated citrated plasma system) do not exert parallel effects.

The mechanisms involved in the potential for lysis in normal vs. abnormally fibrinolytic specimens are not necessarily identical. The dependence of one pathway on a factor which is of no importance to the other may serve to demonstrate the presence of dual mechanisms. Recently Weissbach (*6*) found that the uptake of serotonin by platelets may occur by two different mechanisms, one passive and the other energy consuming. The latter is enhanced by the presence of potassium and phosphate while the former is not. We have previously reported more rapid lysis of normal blood diluted with phosphate than with sodium chloride (*7*). The present studies indicate that potassium further enhances phosphate diluent lysis. The inhibiting effect of calcium, so readily observed in this phosphate diluent-activated system, is not seen in the "undiluted" enzyme-activated system. These latter systems are apparently not dependent on relatively high concentrations of potassium or phosphate. The present studies suggest that the process of retraction and at least one component in the process of fibrinolysis are interdependent. It may be significant that higher concentrations of ε-aminocaproic acid are necessary to produce readily recognizable interference with retraction than with lysis.

## Summary

1. In a clot formed by the addition of thrombin to citrated plasma diluted in phosphate buffer or chloride solution, both fibrinolysis and clot retraction are enhanced by potassium and inhibited by calcium.

2. Both retraction and fibrinolysis of calcium-clotted plasma or thrombin-clotted fibrinogen with added fibrinolytic agent are inhibited by $\epsilon$-aminocaproic acid. Excess calcium will inhibit retraction, but not lysis of plasma clotted with added fibrinolytic agent.

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# Macro and Micro Methods for the Determination of Serum Iron and Iron-Binding Capacity

*Wendell T. Caraway**

**Blood serum is incubated with ascorbic acid and hydrochloric acid to liberate protein-bound iron. Proteins are precipitated with trichloroacetic acid in the presence of chloroform. To an aliquot of clear supernatant is added a solution of tripyridyltriazine and sufficient ammonium acetate to adjust the *p*H between 4 and 5. Absorbance of the blue-colored complex is measured at 590 mμ. Iron-binding capacity is measured by saturation of serum with ferric iron followed by removal of excess iron with magnesium carbonate. Measurement of total bound iron is completed as above. Macro and micro technics for either determination require 2 ml. and 0.1 ml. of serum, respectively. Reliability of the methods has been established by recovery experiments, replicate analyses, and variation of reagent concentrations and incubation times.**

Analytical methods for the determination of serum iron and iron-binding capacity have been reviewed by Ramsay (*25*). The more sensitive reagents available for the colorimetric determination of iron are 4,7-diphenyl-1,10-phenanthroline (bathophenanthroline) and 2,4,6-tripyridyl-*s*-triazine (TPTZ). Molar extinction coefficients of the iron complexes for the two reagents are comparable (22,350 and 22,600 respectively) and are twice that of the complex with 1,10-phenanthroline (*7*). TPTZ was selected for further study of its applicability to the determination of serum iron and total iron-binding capacity (TIBC). The complex with ferrous iron is soluble in acidified aqueous solution. Maximum absorbance occurs at 593 mμ, where background absorbance

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from serum filtrates is less significant. Finally, the reagent has more uniform composition and is less expensive than the commercially available sulfonated bathophenanthroline. The general use of TPTZ has been described (*5*) as well as its application to determination of iron in protein-free filtrates of serum (*36*). The present study is concerned with an evaluation of variables involved in the determination of serum iron and iron-binding capacity and adaptation of the methods to micro technics. The clinical importance of such determinations has been reviewed elsewhere (*11, 20, 25*).

In the proposed method iron is simultaneously reduced and detached from serum protein with the aid of ascorbic acid and hydrochloric acid. Proteins are precipitated by trichloroacetic acid in the presence of chloroform. To an aliquot of water clear supernatant is added a solution of TPTZ and sufficient ammonium acetate to produce an optimum *p*H for color development.

TIBC is determined by incubating serum with excess ferric iron to insure complete saturation of siderophyllin. Excess free iron is removed by treatment with magnesium carbonate powder as described by Ramsay (*24*). An aliquot of clear supernatant is then analyzed for serum iron as a measure of the total iron-binding capacity.

## Materials and Methods

### Glassware

Glassware should be cleaned by soaking in approximately 6N HCl, followed by thorough rinsing with distilled or deionized water. Random contamination with iron is indicated by variable absorbance readings on blanks and standards and by wide discrepancies on duplicate determinations.

### Reagents

*Ascorbic acid, 1% in 0.2N HCl* Prepare by dissolving 0.1 gm. of ascorbic acid in 10 ml. of 0.2N HCl. This solution is stable up to 2 weeks if refrigerated.

*Trichloroacetic acid, 20% (w/v)* Dissolve 20 gm. of crystals in water and dilute to 100 ml. *Caution:* Do not use metal spatulas when weighing out the acid.

*Ammonium acetate, 40% (w/v)* Dissolve 40 gm. of ammonium acetate in water and dilute to 100 ml.

*TPTZ, 0.1%* Dissolve 0.10 gm. of 2,4,6-tripyridyl-*s*-triazine in 2 ml. of glacial acetic acid and dilute to 100 ml. with water. This solution

is stable at room temperature. Do not contaminate TPTZ solution with ammonium acetate as TPTZ will precipitate at higher *p*H. (The reagent used in this study was obtained from G. Frederick Smith Chemical Co., 867 McKinley Ave., Columbus 22, Ohio.)*

*Stock standard iron, 20 mg./100 ml.* Transfer 1.404 gm. of ferrous ammonium sulfate hexahydrate (MW, 392) to a 1000-ml. volumetric flask. Dissolve in about 800 ml. of water, add 0.5 ml. of concentrated sulfuric acid, and dilute to the mark. This solution is stable. Equal results were obtained with standards prepared from pure iron wire or from ferrous ethylene diammonium sulfate (Oesper's reagent; *3*).

*Working standard, 200 μg./100 ml.* Dilute 1.0 ml. of stock standard to 100 ml. with water. It is preferable to prepare this solution fresh on day of use.

*Ferric iron solution, 500 μg./100 ml. in 0.005N HCl.* This is used for total iron-binding capacity only and is prepared as follows: (a) Dissolve 10 gm. of $FeCl_3 \cdot 6H_2O$ in and dilute to 100 ml. with 0.1N HCl. (b) Pipet 0.5 ml. of this mixture into a flask and dilute to 100 ml. with 0.1N HCl. (c) Pipet 5.0 ml. of the resulting mixture into a flask and dilute to 100 ml. with water. This is the final working solution and is stable. The concentration need not be exact.

*Magnesium carbonate, anhydrous, light powder* Adsorptive properties should be checked by substituting water for serum in the method for TIBC. Analysis of the supernatant should show no traces of iron.

## Procedure for Serum Iron

Pipet 2.0 ml. of serum into a small test tube, add 1.0 ml. of ascorbic acid solution, mix, and let stand 5 min. Add 1.0 ml. of 20% trichloroacetic acid and 1 ml. of chloroform. Stopper and shake *vigorously* for 10–15 sec. Centrifuge for 10 min. Carefully decant the supernatant into another small test tube; leave behind a supernatant protein button. The solution should be water clear; if not, it is recentrifuged.

Pipet 2.0 ml. of clear supernatant into a 12-mm. Coleman cuvet or test tube. Set up a blank and standard as follows:

Blank: 1.0 ml. of water + 0.5 ml. of ascorbic acid solution + 0.5 ml. of 20% trichloroacetic acid.

Standard: 1.0 ml. of working standard (200 μg./100 ml.) + 0.5 ml. of ascorbic acid solution + 0.5 ml. of 20% trichloroacetic acid.

To each tube add 0.2 ml. of TPTZ solution and mix. Finally, add 0.2 ml. of ammonium acetate solution and mix very thoroughly. Color develops immediately.

*Should a precipitate form in the solution, add 0.1 ml. concentrated HCl.

Set to zero absorbance with the blank at 590 m$\mu$ and measure the absorbance of the test and standard, preferably within the next 5 min.

$$\frac{\text{Absorbance of test}}{\text{Absorbance of standard}} \times 200 = \text{serum iron } (\mu\text{g./100 ml.})$$

*Note:* The final volume obtained is 2.4 ml. For instruments requiring 3 ml. of solution for measurement, 0.8 ml. of 10% ammonium acetate may be substituted for 0.2 ml. of 40% ammonium acetate in the procedure.

## Procedure for Total Iron-Binding Capacity

This determination should be started some 30 min. before the serum iron so that resulting steps will be coordinated.

Pipet 2.0 ml. of serum to a small test tube, add 4.0 ml. of *ferric* iron solution (500 $\mu$g./100 ml.), mix, and let stand at least 5 min. Add 0.5 gm. of magnesium carbonate powder, stopper, and shake vigorously for 10–15 sec. Let stand for 30 min. but remix thoroughly 4–5 times during this interval. Centrifuge at high speed for 10 min. Pipet 2 ml. of clear supernatant into a small test tube and analyze for serum iron as described in the preceding section.

$$\frac{\text{Absorbance of test}}{\text{Absorbance of standard}} \times 200 \times 3 = \text{TIBC, } \mu\text{g./100 ml.}$$

## Micro Procedures

All volumes are reduced by a factor of 20, and general technics of ultramicrochemistry are applied (*4*). Absorbance is read on the Beckman DU spectrophotometer with ultramicro attachments.

For serum iron, pipet 100 $\mu$l. of serum to the bottom of a 1-ml. centrifuge tube, add 50 $\mu$l. of ascorbic acid solution, mix, and let stand 5 min. Add 50 $\mu$l. of 20% trichloroacetic acid and 0.05 ml. of chloroform. Mix vigorously by drawing the tube back and forth across a test tube rack or with the aid of a Vortex, Jr. mixer (Scientific Industries). Centrifuge for 2 min. in a micro centrifuge at 10,000 rpm. Transfer 100 $\mu$l. of clear supernatant to a 6 $\times$ 50 mm. tube. At this point include a blank consisting of 50 $\mu$l. of water + 25 $\mu$l. of ascorbic acid solution + 25 $\mu$l. of 20% trichloroacetic acid, and a standard, consisting of 50 $\mu$l. of working standard (200 $\mu$g./100 ml.) + 25 $\mu$l. of ascorbic acid solution + 25 $\mu$l. of 20% trichloroacetic acid. To each tube add 10 $\mu$l. of TPTZ solution, mix, then add 10 $\mu$l. of 40% ammonium acetate, and mix thor-

oughly. Transfer to Lowry-Bessey micro cells and measure the absorbance of the test and standard against the blank at 590 mμ in a Beckman DU spectrophotometer.

For TIBC, pipet 100 μl. of serum to the bottom of a 1-ml. centrifuge tube, add 200 μl. of *ferric* iron solution (500 μg./100 ml.), mix and let stand at least 5 min. Add 25 mg. of magnesium carbonate powder, cover the tube with Parafilm,* and mix vigorously for 10–15 sec. Let stand for 30 min. but remix thoroughly 4–5 times during this interval. Centrifuge for 5 min. at 10,000 rpm. Since evaporation is appreciable under these conditions, cover the tube with Parafilm while centrifuging. Pipet 100 μl. of clear supernatant into a 1-ml. centrifuge tube and analyze for serum iron as described in the preceding paragraph. Multiply results by 3 to obtain TIBC.

## Results and Discussion

TPTZ combines with ferrous iron in the ratio 2:1 to form a tridentate chelate ring. The blue color of the complex is developed fully between *p*H 3.4 and 5.8 with maximum absorption at 593 mμ and is stable for hours (*5*). The complex may be extracted into nitrobenzene with slight increase in the molar extinction coefficient. Substances interfering with the determination of iron include $Cu^{++}$, $Co^{++}$, $Ni^{++}$, $Cr^{+++}$, oxalate, nitrite, cyanide, and molybdate. TPTZ forms a precipitate with $Ag^{+}$, $Hg^{++}$, and $Bi^{+++}$. None of these interfering substances would be found in serum in significant amounts, with the exception of $Cu^{++}$. Experiments with standard solutions of cupric sulfate indicate that the absorbance produced by copper under the conditions of the procedure is less than 5% of that produced by an equal weight of iron.

Iron bound to serum siderophyllin is in ferric form (*30*). Binding with serum protein is maximal above *p*H 7 (*33, 34*) although dissociation curves vary depending on the type of buffer present (*29*). Reducing the iron and acidifying the mixture appear to effect rapid dissociation of the bond. The desirability of adding a reducing agent prior to precipitation of proteins has been emphasized by others (*16, 32*). Apparently serum alone, when acidified or heated, must reduce iron to a considerable extent (*17*). In the present method, however, omission of ascorbic acid resulted in slightly lower recoveries.

Independent variations of experimental conditions were investigated. Ascorbic-acid-reagent concentrations were varied between 1

*Marathon Division, American Can Co., Menasha, Wis.

and 5%; hydrochloric-acid-reagent concentrations were varied between zero and 0.6N; incubation times were varied between 5 and 30 min. None of these variations had any effect on the final result. If the 5-min. incubation period was omitted, results were slightly lower. Hydrochloric acid could be omitted entirely but is useful to stabilize ascorbic acid by lowering the *p*H during incubation. Some workers use more concentrated acid and longer periods of incubation to effect complete extraction of iron and imply that low results or incomplete recoveries are obtained otherwise. Actually, use of strong acid at this stage tends to produce chromogens and higher serum blanks which, if not corrected, would cause falsely elevated results (*12, 13, 28*). Higher concentrations of HCl could be used in the present method, provided more ammonium acetate is added for final buffering.

Concentrations of trichloroacetic acid reagent were varied between 10 and 30% with equal results. Chloroform is added to achieve better dispersion and coagulation of protein and to obtain a clear supernatant after centrifuging (*23*). The chloroform also extracts some chromogens from the serum. Supernatant solutions consistently exhibited zero absorbance at 590 m$\mu$. Some workers let the mixture stand for 1 hr. after adding trichloroacetic acid, which provides only for better coagulation of protein (*9*). Others heat the mixture at this stage to insure extraction of all iron. In the present method heating will increase the contribution of iron from any hemoglobin present. Moreover, quantitative recoveries of added iron were obtained without heating.

TPTZ can be substituted for dipyridyl in the method proposed by Ramsay (*23*), but the molar extinction coefficient of the complex is less in mixtures that have been heated. Some attempts were made to apply the TPTZ color reaction directly to serum buffered at *p*H 5 in the presence of ascorbic acid (*10, 19, 29*), but results were low and not reproducible.

The amount of TPTZ employed was shown to be sufficient to develop full color for serum-iron concentrations up to 800 $\mu$g./100 ml. Linear calibration curves were obtained between zero and 500 $\mu$g./100 ml. on either the Coleman or Beckman instruments. Absorbance of the 200-$\mu$g./100 ml. standard at 590 m$\mu$ was approximately 0.28 on the Coleman Jr. Model 6A (12-mm. cuvets) and approximately 0.35 on the Beckman DU (10-mm. light path) against corresponding blanks. Blank absorbance against distilled water usually ranged from 0.02 to 0.03. Although the final color obtained on standards was exceptionally

stable, a slight but significant decrease was encountered in absorbance values on serum filtrates. For this reason it is suggested that readings be taken without delay after development of color.

Optimum concentration of ammonium acetate was determined by discontinuous spectrophotometric and potentiometric titration of standards and protein-free serum extracts prepared as described. Iron was added to pooled serum to approximate final values of 200 $\mu$g./100 ml. Figure 1 illustrates the change in *p*H and absorbance with progressive increase in the concentration of ammonium acetate reagent. Above 30% concentration the absorbance is constant. With 40% ammonium acetate the *p*H is approximately 4.5, and the system is well buffered. The serum extract curve is displaced to the left because the buffer action of serum partially neutralizes the trichloroacetic acid in the system.

Recovery studies were done by analyzing pooled serum to which iron had been added in amounts ranging from zero to 250 $\mu$g./100 ml. Results, shown in Table 1, demonstrate recoveries ranging from 98 to 102% at all levels, even in the presence of appreciable concentrations of neutral fat, bilirubin, and hemoglobin. Serum from one patient with an iron content of 161 $\mu$g./100 ml. was diluted twofold and fourfold with water and analyzed with results of 82 and 41 $\mu$g./100 ml., respectively. This further confirms the completeness of extraction on undiluted serum.

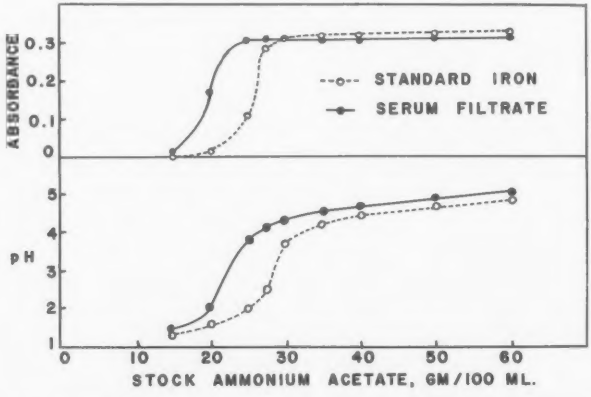


**Fig. 1.** Effect of various concentrations of ammonium acetate reagent on absorbance and *p*H of final reaction mixtures.

**Table 1.** Recoveries of Iron Added to Serum

| *Sample* | *Fe added (μg./100 ml.)* | *Fe found (μg./100 ml.)* | *Fe expected (μg./100 ml.)* | *% recovered* |
|---|---|---|---|---|
| Pooled | 0 | 80 | — | — |
| Pooled | 50 | 128 | 130 | 98 |
| Pooled | 100 | 183 | 180 | 102 |
| Pooled | 150 | 231 | 230 | 100 |
| Pooled | 200 | 276 | 280 | 99 |
| Pooled | 250 | 324 | 330 | 98 |
| Lipemic* | 0 | 90 | — | — |
| Lipemic* | 133 | 218 | 223 | 98 |
| Jaundiced† | 0 | 83 | — | — |
| Jaundiced† | 100 | 185 | 183 | 101 |
| Hemolytic‡ | 0 | 131 | — | — |
| Hemolytic‡ | 100 | 225 | 231 | 98 |

*Neutral fat, 930 mg./100 ml.
†Bilirubin, 40 mg./100 ml.
‡Hemoglobin, 1 gm./100 ml.

Quantitative recovery of iron added to serum does not insure complete extraction and recovery of all native serum iron (*8, 13, 20, 23*). Small but variable quantities of iron may be present in other forms such as ferritin or as hemoglobin iron. Wet-ashing technics will insure recovery of all iron; however, since the clinical and diagnostic significance of serum iron is related to the iron in transport, it is important not to include hemoglobin iron in the measurement. Adequate recoveries of added iron suggest that measurement of native iron attached to siderophyllin is complete.

The macro method for serum iron was compared with the dipyridyl method of Ramsay (*23*) on random sera and on pooled serum containing added iron. Results are shown in Fig. 2. The TPTZ method in this study gave results that averaged 8 μg./100 ml. higher than Ramsay's method. It should be noted, however, that absorbance readings with the dipyridyl method are only one-third of those obtained with the TPTZ method and are in a range subject to greater instrument error.

Hemolysis was found to have less effect on the present method than on those methods which specify heating to precipitate proteins or to liberate iron. Fresh washed red cells were hemolyzed by freezing and added to serum to provide final hemoglobin concentrations of 0.1–0.4 gm./100 ml. With 0.1% hemoglobin, serum iron was increased approximately 5 μg./100 ml. With 0.4% hemoglobin, the increase averaged 13 μg./100 ml. These increases represent the equivalent of about 1% of the available hemoglobin iron. These amounts of hemoglobin

had *no* effect on the TIBC. It would appear, therefore, that some non-hemoglobin iron was contributed from the hemolyzed red cells since it could be removed by treatment with magnesium carbonate. This effect was accentuated when aged red cells were used as the source of contaminating hemoglobin.

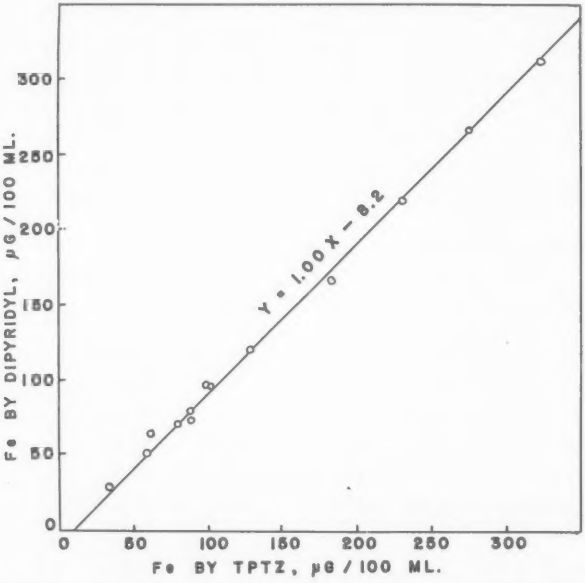


**Fig. 2.** Comparison of dipyridyl and TPTZ methods for serum iron. Equation shown is that of best straight line through points.

Total iron-binding capacity of serum is related to the concentration of a beta$_1$-globulin (transferrin; siderophyllin) capable of binding iron. Immunochemical methods indicate that the average concentration of siderophyllin in serum is 0.27 gm./100 ml. (*14*), corresponding to a TIBC of approximately 340 µg./100 ml. (*20*). The difference between TIBC and serum iron represents the unsaturated iron-binding capacity (UIBC). Various methods have been proposed to measure either UIBC or TIBC. In general, UIBC may be determined by measuring the increase in absorbance produced as iron is added to diluted serum to form the red-colored iron-siderophyllin complex (*15, 26, 33*). Alternately, iron may be added in excess of that required to produce

saturation and the excess iron determined colorimetrically (*10, 19, 27, 29*). Measurement of TIBC involves addition of sufficient iron to insure complete saturation and removal of excess iron with magnesium carbonate (*24*) or ion exchange resin (*18, 22, 31*), or by selective precipitation (*1, 6, 17*). The iron-saturated serum remaining is then analyzed for iron by conventional technics to obtain TIBC directly. Despite its low sensitivity, the method of Rath and Finch (*26*) as modified by Ventura (*33*) or Kaldor (*15*) appears satisfactory for routine determination of UIBC, provided the serum is not hemolytic, lipemic, or jaundiced. For the present study, the method of Ramsay (*24*) was chosen for TIBC as it is not affected by these factors and is more convenient to adapt to micro technics.

The initial steps in the determination of TIBC involve addition of ferric iron solution to serum and removal of excess iron with magnesium carbonate powder. The amount of magnesium carbonate and the time of standing were varied both independently and simultaneously over the range of 0.2–0.8 gm. of magnesium carbonate per 2 ml. of serum and from 15 to 60 min. Samples were shaken intermittently by hand every 5 min. With 0.4 gm. of magnesium carbonate, equal results were obtained at 15-, 30-, or 60-min. incubations. Lesser amounts of magnesium carbonate failed to remove completely excess iron. Larger amounts were satisfactory with 30-min. incubation. Use of 0.5 gm. of magnesium carbonate with 30-min. incubation appears to be optimal, in substantial agreement with Ramsay and with Morgan and Carter (*21*). The magnesium carbonate used in this study was obtained from Matheson, Coleman and Bell. Other preparations are easily checked for adsorptive capacity as described under *Reagents*.

Micro methods for the determination of serum iron have been described that require 20 or 50 $\mu$l. of serum (*2, 35*), but the reagents employed are less sensitive than TPTZ. Macro and micro procedures for serum iron and TIBC as described in this paper were compared by performing 10 replicate analyses on pooled serum. Results, shown in Table 2, indicate comparable precision and show no significant differences between the two methods. Similar results were obtained on random sera in comparison studies. In initial experiments, erratic results obtained on the micro method for TIBC were traced to evaporation losses in the microcentrifuge. When 0.3 ml. of water in an uncovered 1-ml. tube was centrifuged for 5 min. in the Misco centrifuge at 10,000 rpm, a loss of 10% in weight occurred. This would lead to appreciable

**Table 2.** Comparison of Macro and Micro Methods

| | | *Macro (μg./100 ml.)* | | *Micro (μg./100 ml.)* | |
|---|---|---|---|---|---|
| | *N* | *Mean* | *S.D.* | *Mean* | *S.D.* |
| Serum iron | 10 | 96 | 2.3 | 98 | 3.0 |
| TIBC | 10 | 298 | 6.7 | 294 | 4.8 |

concentration of sample but may be prevented by covering the tubes with Parafilm.

To investigate the stability of serum iron and TIBC, one lot of pooled serum was refrigerated at 4° and analyzed in duplicate at intervals for a period of 8 weeks. There was no significant change in either serum iron or TIBC over this interval (Fig. 3). The standard deviations shown indicate probable reproducibility of determinations under routine working conditions.

Normal values for TIBC were reported by Ramsay to range from 250 to 400 μg./100 ml. of serum (*25*). Similar values were reported by Morgan and Carter with use of Ramsay's method (*21*). In this laboratory, analysis of serum from 60 selected patients revealed a mean value of 350 μg./100 ml. with a 95% range of 255–419 μg./100 ml. Other reports in the literature are in substantial agreement with this range (*13, 19, 20, 22*).

Normal values for serum iron are discussed in the review by Ramsay (*25*). No definite range of normal was given in view of the many variables of methodology, diurnal variations, and random fluctuations of serum iron in normal individuals. It is generally accepted that the

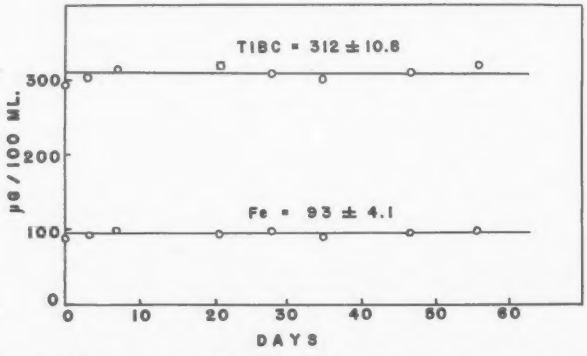


**Fig. 3.** Stability of serum iron and TIBC at 4° showing mean values and standard deviations.

serum iron concentration of normal adult females is some 10–15% lower than in males. A recent collection of normal values from the literature (*19*) includes nine separate studies with mean values ranging from 98 to 173 μg./100 ml. and an over-all normal range from 44 to 238 μg./100 ml. Some of the higher values are subject to criticism on the basis of inadequate serum blank corrections (*28*). It is apparent that excessively wide ranges of normal detract from the value of a test. Our experience with the present method supplemented by a review of the literature has led to a working normal range for serum iron of 65 to 150 μg./100 ml.

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# Simple Open Fusion for an Iodine Screen Test

*Eugene S. Baginski and Bennie Zak*

**A procedure is described for the screening of serum samples for contamination with iodine. It is extremely rapid, obviates any cross-contamination problem, and is not influenced by the organic compounds of raw serum or filtrates. Studies indicate that the open-combustion technic is a reliable screening system for the handling of large numbers of samples.**

SEVERAL TESTS have been proposed for determining the gross contamination sometimes encountered in serums submitted for protein-bound iodine analysis (*1–5*). These screens are all based on the use of the coupled Ce(IV-As(III)) reaction as a detection device. However, ceric ions in strong acid solution represent a powerful oxidation system for many organic compounds (*6*). There is, therefore, a good possibility that these compounds are capable of reducing Ce(IV) to give the appearance of the presence of iodine. It is impossible to differentiate simple Ce(IV) reduction from the iodine catalyzed Ce(IV) and As (III) reaction by measurement of loss of Ce(IV) color alone. Our own experiences with several of these preliminary semiquantitative contamination-exclusion tests has indicated that too many samples were eliminated which subsequent analysis showed to be uncontaminated. We attempted to develop a simple procedure which would yield a reliable screen test with an open-alkaline-combustion technic employing ordinary burners and porcelain crucibles. This extremely rapid process could be used to destroy the organic precipitate containing the iodine without apparent loss of the latter in the conversion to high melting potassium iodide. An important feature of such a system would be the ability to screen large numbers of samples without cross

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contamination. It has been possible to accomplish this, and the results of these experiments will be described here.

## Experimental

### Reagents

*Sodium Hydroxide (0.5N)*

*Zinc Sulfate Stock Solution.* Dissolve 100 gm. of zinc sulfate heptahydrate in a 1-L. volumetric flask and dilute to the mark with water. Standardize against the 0.5N NaOH solution and adjust so that 11 ml. of NaOH is required to neutralize 10 ml. of the zinc solution (*7*).

*Zinc Sulfate Working Solution.* Make up 1 volume of the zinc sulfate stock with 7 volumes of distilled water.

*Sodium Arsenite.* Dissolve 14.34 gm. of reagent-grade sodium arsenite in about 900 ml. of concentrated sulfuric acid, cool, and dilute to the mark with water.

*Ceric Ammonium Nitrate (0.02N).* Dissolve 10.96 gm. of ceric ammonium nitrate in about 500 ml. of distilled water, add 50 ml. of concentrated $H_2SO_4$ and dilute to 1 L. with water.

*Potassium Hydroxide (4N)*

## Procedure

### Manual, Sample

Pipet 1.0 ml. of serum into a crucible (coors No. 170, size 4) containing 8.0 ml. of working zinc sulfate solution and mix well. Add 1.0 ml. of 0.5N NaOH, mix again, and centrifuge the mixture for 5 min. at a moderate speed before decanting the supernatant fluid. Add 1.0 ml. of 4N KOH and mix thoroughly until the precipitate dissolves. Dry the crucible in an oven set at 100°, a step which only takes about 30 min. because of the geometry of the crucible. Place the crucible in a slanted position on a triangle wire above a Fisher Burner and keep the flame at low temperature until the residue becomes black (the residue usually burns with a flame at this point). Increase the flame of the burner to its maximum and rotate the crucible if necessary until all the content becomes yellow and no more carbon is visible (about 3–5 min.). Turn the flame off and let the cruicble cool a little before placing it on an asbestos pad to come to room temperature. Pipet 6.0 ml. of As(III) solution into the residue and mix until there is no further effervescence. Centrifuge at high speed for 5 min. Take out a 5.0-ml. aliquot by carefully tipping the crucible on its side in order to avoid pipeting the precipitate and transfer the aliquot into a test tube. Place the tube

in a water bath at 35° for 10 min. Add 1.0 ml. of Ce(IV) solution to each tube and then observe the tubes after 20 min. of incubation. Ordinarily, when a sample is grossly contaminated, it decolorizes almost on contact with the Ce(IV). The other contaminated samples are colorless at various intervals up to 20 min., usually much less. These samples are then removed from the day's run.

## Discussion and Results

The original stated purpose of this investigation was to develop a reliable screening system. Three phases of study were felt to be necessary to show reliability, and these phases could be listed as follows:

1. The analysis of known contaminated samples as well as clean samples which were artificially contaminated
2. The study of the residual melt to prove that the catalysis was due to iodine
3. Tracer studies.

### Contaminated Samples

Throughout a period of 18 months' study during which over 1300 samples were analyzed, some samples were found to be grossly contaminated. In almost every instance, previous histories showed that these patients had received large amounts of iodine in some form. Samples were selected from patients who had recently received this kind of medication, and again they were found to be grossly contaminated. Then, a series of serums were set up where an excess of iodate was added prior to drying and ashing. The effect of the reaction is described as follows:

#### Addition of 100 μg. per 1/100 ml.

Five samples were processed, and the absorbance readings ranged from 0.07 to 0.10 after 9 min., 0.01–0.02 after 13 min. and completely colorless after 17 min.

#### Addition of 200 μg. per 1/100 ml.

Ten samples were analyzed. After 5 minutes the readings ranged from 0.05–0.08, and after 10 min., a complete decolorization was spectrophotometrically apparent.

### Residual Melt Experimentation

Several serum samples were ashed in duplicate using the open-fusion technic. After the fusion was complete and the samples were

cooled for subsequent handling, acid arsenic was added to both melts to effect solution. A trace of mercury salt was added to one of each of the duplicates, and then the Ce(IV)-As(III) reaction was carried out. One aliquot of each set retained its catalytic activity while the sample containing mercury was even less decolorized than a reagent blank. This experiment showed good indirect evidence that the catalytic activity was due to iodine since this would be mercury inhibited.

If mercury had been present in the samples before fusion, it would have been lost because of the high temperature of the fusion process. No mercury inhibition has been encountered in this process in the more than 2 years during which this entire study was carried out.

### Tracer Study

Twelve serum samples were treated with precipitating agents. A drop of albumin containing radioactive iodine and 1 ml. of 4N KOH were added. A count was made with a scintillation counter after oven-drying the samples. The samples were then ashed with Fisher Burners for 5 min. and recounted. The results showed two samples with a final count indicating complete recovery, whereas the remaining samples with one exception (63%) ranged between 80% and 96%. We have no explanation for this situation and cannot solve it in terms of the possibilities of variable geometry, loss, or some other phenomenon. However, these recoveries would be more than adequate for a screen test as described.

## Addendum

Since this work was completed, we have successfully experimented with electrothermal Bunsen burners in place of Fisher burners.

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# A Mercurimetric Chloride Determination Suitable for Icteric Sera

*B. Fingerhut and W. H. Marsh*

**A modification of the direct mercurimetric determination of serum chloride suitable for icteric sera is presented. The method is simple, rapid, and accurate. No deproteinization of the sample is required. The results agree well with the standard protein-free filtrate procedure of Schales and Schales.**

THE DETERMINATION of serum chlorides by the titrimetric method of Schales and Schales (*2*) is still widely used in clinical chemistry laboratories. The original authors recommended the use of a protein-free filtrate for ease of endpoint detection. They also suggested that the chloride determination could be performed directly upon serum without loss in accuracy. Actually, the latter procedure is found to be true only for sera which are not highly pigmented. The presence of bilirubin or other chromogens in high concentration interferes with the accuracy of the determination by obscuring the endpoint. For reliable results, highly jaundiced sera should be deproteinized to remove the bilirubin along with the protein.

We have found it advantageous to modify the Schales and Schales method so that the effect of chromogenic substances could be minimized without the necessity of filtrate preparation. A procedure is presented which uses 0.2-ml. samples and is a simple, direct titrimetric method yielding reliable results for both icteric and nonicteric specimens. The modification involves the brief heat treatment of the sample with 3% hydrogen peroxide to destroy serum pigments.

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## Procedure

### Method A: Schales and Schales

Make a 1:10 dilution of serum using conventional tungstic acid deproteinization. To 2.0 ml. of the supernatant solution add 2 drops of 0.5% S-diphenylcarbazone and titrate to the violet endpoint with standardized mercuric nitrate.

### Method B: Proposed Method

Add to a 4 × ½-in. test tube 0.2 ml. serum and 0.2 ml. 3% hydrogen peroxide. Place tube in a boiling-water bath for 30–60 sec. Add 1.5 ml. water, 2 drops 0.5% S-diphenylcarbazone, and 1 drop n-octyl alcohol (antifoam). Insert a fine capillary tube connected to a source of compressed air to serve as a mixing device. Titrate with mixing to a violet endpoint.

To standardize the mercuric nitrate solution repeat the procedure with 0.2 ml. of standard NaCl solution.

### Method C: Control of Method B

Proceed as in Method B but omit the heating with 3% $H_2O_2$.

## Results

The results using Methods A and B with sera of varying bilirubin content are shown in Table 1. Statistical analysis of the differences between the two procedures yields a standard deviation of ± 1.35 mEq./L. chloride. Calculation of t (*3*) from these data for 95% probability indicates that there is no characteristic difference between the two methods.

The precision attained by direct titration when an icteric serum was analyzed 10 times for chloride content with and without peroxide addition is presented in Table 2. The standard deviation of ± 1.56 mEq./L. without peroxide, when compared to the standard deviation of ± 0.47 mEq./L. with peroxide, clearly shows that greater reproducibility is obtained with peroxide addition.

Recovery experiments using sera of normal and moderately elevated bilirubin content, were performed to provide an indication of the accuracy of Methods B and C. Results of triplicate determinations directly on sera, with and without the use of peroxide appear in Table 3.

**Table 1.** Results with Protein-free Method of Schales and Schales (Method A) and Direct Peroxide Method (Method B)

| *Bilirubin (mg./100 ml.)* | *Chloride (mEq./L.)* | |
|---|---|---|
| | *Method A* | *Method B* |
| 0.7 | 105.1 | 104.5 |
| 0.5 | 104.2 | 103.6 |
| 0.3 | 104.5 | 103.4 |
| 17.6 | 79.3 | 76.8 |
| 11.3 | 110.4 | 107.8 |
| 21.2 | 103.8 | 104.5 |
| 8.7 | 105.1 | 102.7 |
| 0.5 | 99.7 | 101.8 |
| 5.2 | 88.2 | 86.7 |
| 2.1 | 111.7 | 109.8 |
| 0.6 | 100.4 | 99.8 |
| 4.5 | 106.3 | 104.4 |
| 0.8 | 105.7 | 104.1 |
| 0.5 | 109.2 | 107.7 |
| 0.9 | 104.3 | 104.7 |
| 0.6 | 120.0 | 121.8 |
| 0.3 | 100.6 | 101.7 |
| 0.6 | 93.9 | 93.2 |
| 26.2 | 101.5 | 103.0 |
| 5.2 | 103.1 | 105.2 |
| 19.0 | 88.5 | 88.3 |
| 3.8 | 103.7 | 102.3 |
| 2.4 | 107.0 | 105.8 |
| 3.5 | 98.0 | 100.5 |
| 1.0 | 106.4 | 106.3 |
| 0.7 | 102.0 | 104.1 |
| 0.5 | 108.5 | 108.6 |
| 1.0 | 94.3 | 95.7 |
| 0.7 | 103.3 | 102.3 |
| 0.3 | 136.6 | 136.3 |
| | Average 103.51 | 103.26 |

As can be seen, the recoveries ranged from 100.1 to 100.8% when peroxide was added, and from 99.8 to 101.7% without peroxide.

## Discussion

The reaction between hematin or hemoglobin and peroxide has been previously reported (*1*). In the absence of catalase, a colorless oxidation product (propentdyopent) is formed. Further treatment with dithionite in alkaline solution produces a red product (pentdyopent). The same sequence of reactions appears to take place with serum pig-

**Table 2.** Precision of Chloride Determination in Presence and Absence of Hydrogen Peroxide

| | *Chloride (mEq./L.)** | |
|---|---|---|
| | *Method B (peroxide present)* | *Method C (peroxide absent)* |
| | 102.4 | 101.4 |
| | 103.3 | 102.3 |
| | 103.6 | 101.6 |
| | 103.6 | 103.2 |
| | 103.3 | 100.6 |
| | 103.3 | 103.6 |
| | 104.0 | 104.0 |
| | 103.6 | 104.5 |
| | 103.3 | 104.5 |
| | 104.0 | 105.3 |
| Average | 103.4 | 103.1 |
| S.D. | ±0.47 | ±1.56 |

*Ten determinations were done on each sample.

**Table 3.** Results of Triplicate Chloride Recovery Experiments Directly on Sera

| *Specimen* | *Bilirubin conc. (mg./100 ml.)* | *Method C— $H_2O_2$ absent (mEq./L. Cl)* | *Theoretical (mEq./L. Cl)* | *Recovery (%)* | *Method B— $H_2O_2$ present (mEq./L. Cl)* | *Theoretical (mEq./L. Cl)* | *Recovery (%)* |
|---|---|---|---|---|---|---|---|
| Serum A | 9.5 | 115.7 | | | 115.2 | | |
| 1 part A + 1 part 100 mEq./L. NaCl | | 108.9 | 107.8 | 101.7 | 108.5 | 107.6 | 100.8 |
| 2 parts A + 1 part 100 mEq./L. NaCl | | 111.5 | 110.4 | 101.0 | 110.9 | 110.4 | 100.3 |
| Serum B | 0.6 | 90.5 | | | 90.1 | | |
| 1 part B + 1 part 100 mEq./L. NaCl | | 95.1 | 95.2 | 99.9 | 95.3 | 95.1 | 100.2 |
| 2 parts B + 1 part 100 mEq./L. NaCl | | 93.5 | 93.7 | 99.8 | 93.5 | 93.4 | 100.1 |

ments. A red coloration, presumably pentdyopent, was produced by heating peroxide-decolorized sera with alkaline dithionite.

When 0.2 ml. of 0.5 N NaOH is added to a peroxide-serum mixture, an increased rate of decolorization occurs at room temperature. However, in order to complete the titration with mercuric nitrate the addition of acid would be required to neutralize the added alkali. When the alkali is omitted, as is proposed in the present modification, the short heating period serves to decolorize almost completely the serum pigments. The faint green color in some samples, presumably due to biliverdin, offers no interference to detection of the endpoint.

The results in Tables 2 and 3 indicate the superior precision and recoveries with the peroxide method (Method B) as compared to the control with peroxide omitted (Method C). The data in Table 1 give further support for the reliability of the proposed method. Thirty results obtained using Folin-Wu filtrates averaged 0.25 mEq./L. higher than the direct procedure with peroxide addition. No clinical significance could be attached to this slight difference. It does not represent loss of chloride in the peroxide method since standards heated at 100° with 10 times the concentration of peroxide for 5 times the time interval used in the analytical procedure showed no chloride change.

In the presence of protein, Schales and Schales (*2*) obtained apparent chloride values from 1–2 mEq./L. higher than in its absence. Annino (*4*) and Rice (*5*) reported apparent chloride values of 6–8 mEq./L. higher in the presence of protein, with globulin producing a greater effect than albumin (*4*). This latter effect might be explained by the higher tyrosine content of globulins, (*6*) supplying more mercury-reactive material than is found in albumin.

The silver-reactive groups present in protein are less reactive than those reacting with mercury ions as evidenced by the work of Annino (*4*) and Cotlove (*8*), and represent primarily —SH groups. These give rise to results higher than normal by about 0.6 mEq./L. (*7, 8*). The removal of most of these interferences appears to be accomplished by peroxide oxidation, as proposed in the present method.

The presence of peroxides in the ether modification of Rice (*5*) may explain the removal of pigment and protein interferences; however, a greater affinity of the mercury for indicator occurring in the ether layer would also reduce interference by pigment and protein in the aqueous phase. The use of peroxide in the present method eliminates ether hazard and gives better correlation (*5*). The noncritical nature of peroxide concentration and heating time should allow this proposed simple modification to be readily incorporated in the Schales and Schales method, thereby increasing the ease of endpoint detection and precision.

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# Variation in Excretion of Certain Amino Acids With Age

*R. Stambaugh, D. T. Davidson, Jr., and J. R. Elkinton*

**By relating amino acid excretion to creatinine excretion in a specimen of urine from fasting patients, the normal variation of cystine, glutamine, glycine, and alanine excretion in relation to creatinine excretion and the age of the individual has been defined. No amino acid excretion differences between the two sexes were detectable. In general, the excretion levels for these four amino acids were found to be highest in infancy and decreased markedly with age. However, this decrease is not a uniform or gradual one. The greatest decrease occurs between the 3–5-year and 6–8-year groups, and the excretion levels again increase slightly between the 9–11-year and 12–14-year groups.**

THE ASSESSMENT of pathologic patterns of excretion of several amino acids requires an extensive quantitative knowledge of the normal patterns in human subjects without known metabolic disease. To date, this knowledge is inadequate both as to total amounts of each amino acid excreted per unit time and as to variation with sex, age, and size.

In 1949 Herman *et al.* (*1*) measured many of the amino acids in specimens of urine collected over periods of 24 hr. Because of the obvious difficulty in obtaining such accurate collections, other investigators have attempted to relate the excretion rates of amino acids to that of total nitrogen (*2–4*). Total nitrogen excretion varies greatly, and amino acid excretion but slightly, with daily variation in protein intake (*4, 5*); exceptions to this are histidine and 1-methylhistidine (*4*). For these reasons the excretion rate of creatinine, which is more closely related to the size of the total metabolizing lean body mass (*6*),

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would appear to be a better reference point for quantitation of the rate of excretion of the several amino acids. This was done in 1957 by Fowler *et al.* (*4*), who concluded that no significant changes took place after the age of 10. However, more data were needed to define the variation of amino acid excretion with age and sex.

In this investigation we have attempted to define more accurately the relationships of rates of excretion of four of the major amino acids per unit of creatinine excreted to age and sex. Enough human subjects were used to determine the distribution of normal values in a series of age groups up to 30 years of age and in both sexes. In general, the excretion levels for these four amino acids were found to be the same in both sexes and to be highest in infancy and to decrease markedly with age.

## Experimental Material and Methods

### Subjects

The experimental subjects were 120 healthy males and 40 healthy females, the majority of whom were mentally retarded students of the Elwyn School between infancy and 30 years of age; no clinical or biochemical evidence was present of any of the known inborn errors of metabolism.

### Collection and Preparation of Specimens

Fasting urine specimens were collected in the morning before breakfast and before any appreciable amount of physical activity. The specimens were preserved for analysis by refrigeration and by a layer of toluene on the surface of the specimen. Amino acid analyses were carried out within 7 days after collection. Ten-milliliter samples were centrifuged to remove the sediment. Six milliliters of the supernatant solution was transferred to a RSCo Model A-1930 electric desalting apparatus. Sufficient voltage was applied to give an initial current of 0.6 amp. When the current decreased to 0.5 amp, the specimen was pipetted off and recentrifuged. The supernatant solution was used for the creatinine and amino acid analyses. The creatinine content of the desalted specimen was determined photometrically by its color reaction with picric acid in alkaline solution (*7*, *8*).

### Chromatography of the Amino Acids

A specimen of the desalted fasting urine of a volume to contain 65–130 $\mu$g. of creatinine was applied to Whatman No. 1 chromatography

paper (18¼ × 22½ in.) for two-dimensional paper chromatography. The air-dried spot was then wet once with 30% hydrogen peroxide, which converts the cystine to cysteic acid and the methionine to methionine sulfoxide. When the spot had dried the amino acids were separated by the method of Levy and Chung (*9*), using n-butanol:acetic acid:$H_2O$ (4:1:5, v/v/v) and m-cresol:phenol:borate buffer, 0.114 M, *p*H 9.3 (25:25:7, w/w/v), for development of the two-dimensional paper chromatogram. The 0.114 M borate buffer represents a modification of their original method, which used a 0.057 M borate buffer.

#### Quantitation of the Amino Acids on the Paper Chromatogram

The dried papers were developed by dipping in a modified ninhydrin solvent consisting of 200 mg. of ninhydrin in acetone:acetic acid:collidine (100:10:1, v/v/v) and heating in an oven for one hour at 60°. The spots were immediately cut out and eluted for 1½ hr. at room temperature with 4.0 ml. of a solution containing 0.5% $Cu(NO_3)_2$:10% nitric acid:95% ethanol (5:1:14, v/v/v).

At the end of 1½ hr., the solutions were filtered into 1.0-cm. cuvettes, and the absorbance of the solution was determined at 515 mμ in a Beckman DU spectrophotometer using a reagent blank.

The concentration of each amino acid was calculated from the absorbance and from a standard curve for each amino acid. These standard curves were prepared by chromatographing known quantities of each amino acid with the same technics that were used for the urine specimens. Repeated determinations of known quantities of these four amino acids demonstrated an average error of ± 7.3% for alanine, ± 6.2% for glutamine, ± 10.3% for glycine, and ± 10.2% for cystine.

## Results

The results are presented in Table 1 and in Fig. 1–4.

The excretion of all four amino acids relative to that of creatinine fell with increasing age. For each amino acid the greatest decrement occurred between the 3–5-year group and the 6–8-year group ($P < 0.01$), and there was a slight rise or leveling off between the 9–11-year and the 12–14-year groups. The widest variations occurred with glycine.

Our results for glycine and alanine excretion agree well with the few values obtained by Fowler *et al.* (*4*). Our values for cystine excretion appear to be slightly higher than theirs, and they did not determine the level of glutamine excretion in their patients.

**Table 1.** Rates of Excretion of Glycine, Alanine, Cystine, and Glutamine

| Age group (yr.) | Excretion rates (μM/gm. creatinine) | | | | | | | |
|---|---|---|---|---|---|---|---|---|
| | Glycine | | Alanine | | Cystine | | Glutamine | |
| | Mean s.d.* | n† | Mean s.d. | n | Mean s.d. | n | Mean s.d. | n |
| 0–2 | 2,597 ± 807 | 9 | 829 ± 296 | 9 | 425 ± 136 | 9 | 950 ± 323 | 9 |
| 3–5 | 2,436 ± 843 | 13 | 886 ± 352 | 13 | 331 ± 103 | 12 | 943 ± 289 | 13 |
| 6–8 | 1,251 ± 737 | 11 | 492 ± 151 | 10 | 211 ± 89 | 12 | 523 ± 233 | 12 |
| 9–11 | 993 ± 688 | 33 | 459 ± 178 | 31 | 181 ± 52 | 33 | 535 ± 224 | 31 |
| 12–14 | 1,084 ± 518 | 40 | 478 ± 140 | 37 | 190 ± 65 | 40 | 550 ± 164 | 39 |
| 15–19 | 1,147 ± 624 | 42 | —‡ | | 145 ± 69 | 42 | 503 ± 206 | 42 |
| 20–29 | 1,119 ± 597 | 11 | —‡ | | 133 ± 37 | 11 | 476 ± 115 | 11 |

*Standard deviation.
†Number of subjects.
‡Not amenable to statistical analysis since the concentration in the specimens occasionally fell below the minimum detectable level of 130 μM/gm. creatinine.

There appeared to be no difference in the excretion levels of these amino acids between the two sexes.

## Discussion

The excessive excretion of amino acids in the urine is a manifestation of aberrant cellular function. It may result from deranged intermediary amino acid metabolism in various organs and from impaired renal tubular reabsorption. For the clinician, patterns of urinary amino acid excretion may provide essential clues in diagnosis, and in genetic studies the excretion patterns provide valuable information for the elucidation of metabolic defects occurring in obscure syndromes. Our interest is primarily in the latter area, which includes inborn errors of metabolism, especially those associated with mental retardation.

However, the normal ranges of amino acid excretion have never been accurately defined, and one is faced with the problem of identifying pathologic excretion patterns without a sufficient knowledge of the normal values. This is especially true when one tries to evaluate the excretion levels of the individual amino acids—that is, a "specific" aminoaciduria. In this study we have accumulated sufficient data for determining the range of normal variation of amino acid excretion in different age groups for four of the amino acids. Except for cystine, our results are in good agreement with the data of Fowler *et al.* (*4*). Our cystine-to-creatinine ratios, shown in Fig. 3 and Table 1, appear to

be slightly higher than those reported by Fowler *et al.* (*4*). This difference might be explained by the fact that they used 24-hr. urine specimens for analysis, while we used the morning specimen collected before breakfast. However, it seems more likely that their values are too

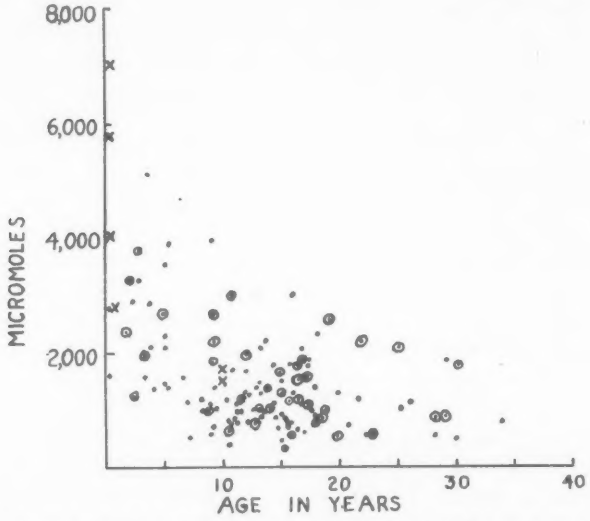


**Fig. 1.** Micromoles of glycine excreted per gram of creatinine. Dots represent males, and circles, females. Values reported by Fowler *et al.* (*4*) are represented by crosses.

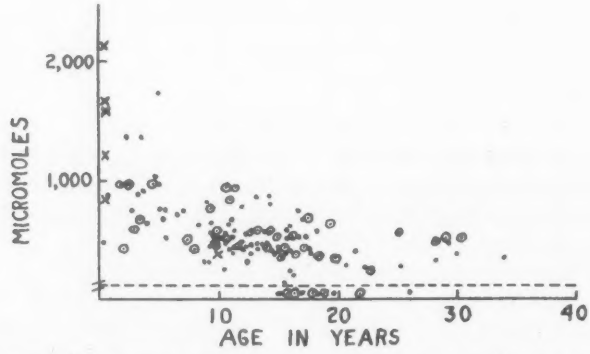


**Fig. 2.** Micromoles of alanine excreted per gram of creatinine. Symbols are as in Fig. 1. Dotted line represents minimum detectable concentration of alanine by these technics.

low due to a partial loss from auto-oxidation to cysteic acid during the isolation procedure. Prior conversion of cystine to cysteic acid with hydrogen peroxide eliminates the loss due to auto-oxidation.

The importance of accurately relating the amino acid excretion to age, in addition to creatinine excretion, is evident in the excretion of cystine by an 18-year-old cystinuric, as shown in Fig. 3. If this value were found for a one-year-old child, there would be considerable doubt that the child had cystinuria; for an 18-year-old girl the abnormality is unequivocal.

The explanation for the sharp statistical decrease between the 3–5 and 6–8-year age groups, followed by a secondary slight rise in excre-

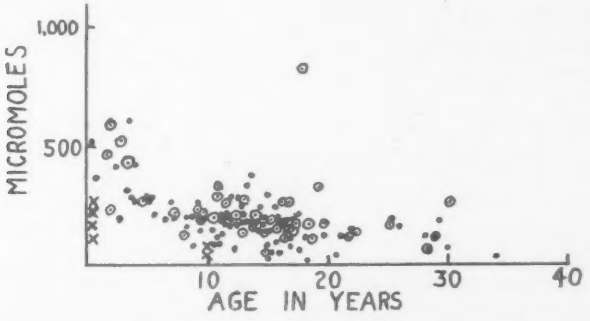


**Fig. 3.** Micromoles of cystine excreted per gram of creatinine. Symbols are as in Fig. 1. Cystine excretion level of an 18-year-old female cystinuric is recorded for comparison with normal values.

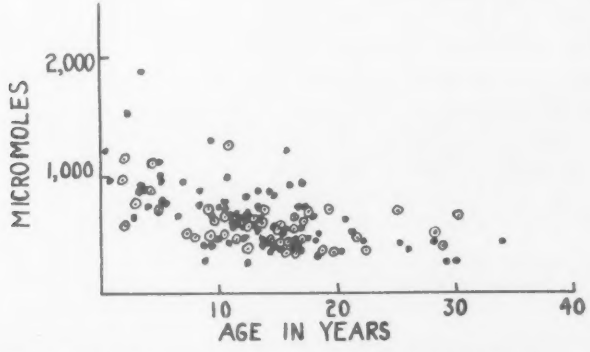


**Fig. 4.** Micromoles of glutamine excreted per gram of creatinine. Symbols are as in Fig. 1.

tion in the 12–14-year age group remains obscure. Although the data of Fowler *et al.* (*4*) were inadequate to detect such a decrease in these four amino acids, it is of interest that they observed a decreased excretion of taurine between infancy and the age of 10 years. It is well known that the skeletal musculature grows more rapidly than the body as a whole between the ages of 5 and 10 (*10*), and one might speculate that the decreased amino acid excretion levels result from increased utilization for muscle growth.

Whatever the explanation for the variation of the excretion rates of glycine, alanine, cystine, and glutamine, relative to creatinine, our data do provide in each instance and for each age group a "normal" population of values with which any single determination in an unknown patient may be compared. This should be of great diagnostic and research use in the assessment of these particular amino acids in "overflow" or renal aminoacidurias (*5*, *11*).

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# A Study of the Accuracy and Precision of Clinical Chemistry Determinations in 170 Canadian Laboratories

*David B. Tonks*

**Lyophilized samples of two serum pools, prepared commercially, were sent to 200 Canadian clinical chemistry laboratories for estimations of total cholesterol, sodium, chloride, glucose, nonprotein or urea nitrogen, total protein, and inorganic phosphorus. The purpose of this evaluation study was to determine if these laboratories, as a group, were performing satisfactorily, and to help individual laboratories to evaluate their performances and detect gross errors in their results.**

**Values were reported from 170 laboratories. A summary of the complete data is presented in the form of tables, scatter diagrams, and frequency charts. It was found that there was a lack of accuracy and precision in many laboratories. Over 40% of the 3762 values reported fell outside of the allowable limits of errors and therefore were classified as unacceptable. These results indicated a need for improved performances in many of the participating laboratories.**

SINCE 1947 a considerable number of evaluation studies of the accuracy of clinical chemistry determinations have been carried out in various countries (*1–5*). They have shown that there is a need for improved performance in many laboratories. Canadian experience has been gained from a number of surveys conducted by Tonks and Allen from 1952 to 1954 (*6, 7*). Although these Canadian studies proved to be useful, they were limited in scope and showed the need for a more

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The study reported here was a personal research project and was not conducted on behalf of any society, association, company, or institution. It was supported financially by the Warner-Chilcott Laboratories of Canada and the U.S.A. The help and cooperation of the participants is also gratefully acknowledged.

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representative national study which would include a larger number of laboratories and which would therefore emphasize much more strongly the need for increased accuracy and precision in clinical chemistry determinations. Such a study has been completed and is described here. One hundred and seventy laboratories from across Canada carried out 3762 analyses for eight components in two freeze-dried serum specimens. Allowable limits of error were set for each determination. Using these limits, over 40% of the results were classified as inaccurate and unacceptable. An estimate of precision was also possible since two specimens were analyzed. The results indicated a serious lack of precision as well as accuracy in many laboratories.

## Method

Invitations to participate in the survey were sent to 226 laboratories. Included were the laboratories in all Canadian hospitals of 100 beds or over, and a few commercial and government laboratories where clinical chemistry analyses were performed routinely. About 200 affirmative replies were received, and subsequently two specimens, in the form of freeze-dried serums,* were sent to each participant, with complete instructions and report forms. After reconstitution, each laboratory had 10-ml. volumes of the specimens for analysis. Aliquots of the same specimens were sent to each laboratory, but the sample bottles were numbered differently in each case. The bottles had no other markings and were packaged in plain boxes.

Participants were asked to analyze both specimens (A and B) for sodium, chloride, total protein, glucose or total reducing substances, urea nitrogen or nonprotein nitrogen, and phosphorus, and one specimen (B) for cholesterol. However, they were instructed to do only determinations which were performed routinely. Instructions were also given to carry out as many analyses as possible in duplicate. The values obtained, with information concerning the methods used, were sent directly to the author for coding and tabulation. When all data had been received, the stated values for each component, the means of the values reported, and the proposed allowable limits of error were furnished to each participant.

*Special lots of Versatol and Versatol-A, provided by the Warner-Chilcott Laboratories of Canada, were used for the study. These products were prepared in the usual manner by adding weighed amounts of the various components to large pools of serum after these components have been either completely removed or reduced to a known constant level by selective dialysis. Samples of each batch were tested by independent laboratories. They proved to be ideal specimens for the survey since they were stable, had no added preservatives, and contained accurately known concentrations of the various components.

The allowable limits of error were calculated by means of an empirical formula which the author has found to be useful. This formula is based upon the premise that errors should not exceed one-quarter of the normal range. This formula is as follows:

$$\text{Allowable limits of error (in \%)} = \pm \frac{(\frac{1}{4} \text{ of the normal range})}{(\text{mean of the normal range})} \times 100\%.$$

If the normal range for sodium is taken to be 135-145 mEq./L., then the allowable limits of error calculated by this formula are ± 1.8%. The maximum limits for any determination, however, were set at ± 10%, even though in some cases those calculated by the above formula exceeded this figure. For this study, then, the allowable limits of error have been established as follows: for sodium, ± 1.8%; for chloride, ± 2%; for total protein, ± 7%; for glucose, total reducing substances, phosphorus, urea nitrogen, NPN, and cholesterol, ± 10%. These limits are comparable with those used by other authors in similar studies (*1, 4, 8–11*).

An evaluation of the accuracy with which each component was determined was obtained by calculating the percentage of results which fell outside of these allowable limits of error. To evaluate precision, use was made of standard deviations, scatter diagrams, and frequency charts. Also coefficients of variation for the values reported for the two samples were compared with the coefficients calculated from the ratios of the values.

The tabulation of the cholesterol results, which incidentally were from one sample only, presented a problem because of the many different methods used. It was decided to divide the data into four groups as shown in Table 1. Since only the absolute concentration of cholesterol was furnished by the manufacturer, it was necessary to determine "correct" values for the four groups in the author's laboratory. These determined values were used in place of the stated value for all calculations.

## Results and Discussion

Of the 276 laboratories which were invited to participate, 170, or 61.7%, completed the study. The results obtained are summarized in Table 1. The last two columns give the percentages of values classified as unacceptable, that is, the percentages of the values which fell outside of the ranges calculated from the suggested allowable limits of

**Table 1.** SUMMARY OF RESULTS

| *Component* | *Sample* | *Stated value** | *No. of values reptd.* | *Mean value* | *Median value* | *Range of values* | *S.D.* | *Coef. of vari.* | *Allowable limits of error (%)* | *Percentage of unaccept. values* | |
|---|---|---|---|---|---|---|---|---|---|---|---|
| | | | | | | | | | | *Based on stated value* | *Based on mean* |
| Total red. substances | A | 100 | 239 | 95.6 | 96.0 | 62 –125 | 9.20 | 9.2 | ±10 | 27.2 | 27.2 |
| | B | 220 | 230 | 214 | 217 | 148 –285 | 19.1 | 8.9 | ±10 | 19.1 | 22.2 |
| "True" glucose | A | 86 | 90 | 84.6 | 84.0 | 63 –115 | 10.2 | 12.1 | ±10 | 34.4 | 32.2 |
| | B | 197 | 90 | 193 | 190 | 154 –240 | 16.8 | 8.7 | ±10 | 20.0 | 17.8 |
| Inorganic phosphorus | A | 3.70 | 275 | 3.90 | 3.90 | 1.85– 9.00 | 0.63 | 16.1 | ±10 | 22.0 | 22.6 |
| | B | 8.0 | 263 | 7.88 | 8.10 | 2.8 – 11.0 | 1.12 | 14.2 | ±10 | 22.4 | 25.4 |
| Total protein | A | 6.90 | 320 | 7.19 | 7.20 | 4.15– 10.1 | 0.63 | 8.6 | ± 7 | 40.0 | 26.3 |
| | B | 4.4 | 310 | 4.92 | 4.90 | 3.7 – 6.7 | 0.45 | 9.2 | ± 7 | 71.5 | 30.3 |
| Sodium | A | 139 | 260 | 141 | 140 | 122 –205 | 5.98 | 4.2 | ± 1.8 | 53.5 | 45.4 |
| | B | 126 | 272 | 126 | 125 | 110 –155 | 5.49 | 4.4 | ± 1.8 | 53.7 | 53.7 |
| Chloride | A | 102 | 299 | 101.8 | 101.5 | 81.2 –130 | 4.98 | 4.9 | ± 2 | 41.8 | 41.8 |
| | B | 89 | 290 | 92.2 | 91.8 | 79.1 –115 | 4.78 | 5.2 | ± 2 | 75.0 | 53.5 |
| Urea nitrogen | A | 12.1 | 196 | 13.5 | 13.0 | 3.4 – 40 | 4.13 | 31.6 | ±10 | 55.0 | 52.0 |
| | B | 29.5 | 195 | 31.3 | 30.0 | 3.6 – 85 | 7.25 | 23.1 | ±10 | 47.6 | 46.1 |
| NPN | A | 27 | 122 | 33.6 | 33.5 | 10.8 – 62.0 | 7.58 | 22.5 | ±10 | 74.5 | 64.6 |
| | B | 47 | 121 | 49.7 | 49.0 | 20 – 76 | 8.30 | 17.6 | ±10 | 47.1 | 43.0 |
| Cholesterol (S & S) | B | 90† | 23 | 99.9 | 95.0 | 89.7 –140 | 13.9 | 13.9 | ±10 | 39.0 | 30.4 |
| Cholesterol ($FeCl_3$) | B | 103† | 52 | 114.8 | 111 | 86.5 –180 | 20.2 | 17.6 | ±10 | 53.9 | 53.9 |
| Cholesterol (Bloor) | B | 106† | 139 | 123.2 | 118 | 42.4 –263 | 34.4 | 28.0 | ±10 | 70.5 | 61.1 |
| Cholesterol (Direct LB) | B | 106† | 97 | 126.5 | 128 | 83 –201 | 26.4 | 20.9 | ±10 | 83.5 | 67.0 |
| | | AVERAGE | | | | | | | | 47.6% | 40.9% |

*Total red. substances, glucose, P, urea N, NPN, and cholesterol values are expressed as mg./100 ml.; protein as gm./100 ml.; Na and Cl as mEq./L.

†For cholesterol, the values determined by the author by four representative methods, have been used in place of the stated value of 86, for all calculations.

**Table 2.** CLASSIFICATION OF ALL VALUES ACCORDING TO THEIR MAGNITUDE OF ERROR

| | *For errors based on content* | | *For errors based on means* | |
|---|---|---|---|---|
| *Classification of errors* | *No. of values* | *% of values* | *No. of values* | *% of values* |
| Error < allowable error | 1971 | 52.4 | 2223 | 59.1 |
| Error > allowable error | 1791 | 47.6 | 1539 | 40.9 |
| Error > 1½ × allowable error | 1290 | 34.3 | 970 | 25.8 |
| Error > 5 × allowable error | 652 | 17.3 | 476 | 12.6 |
| Error > 10 × allowable error | 184 | 4.9 | 100 | 2.7 |

error. These ranges were estimated both from the mean values and from the absolute or stated values of the specimens. For total reducing substances in Specimen A, for example, the stated and mean values were 100 mg.% and 95.6 mg.%, respectively, and since the allowable limits of error were ± 10%, the acceptable range of values were calculated to be 90–110 mg.% and 86.4–104.2 mg.%, respectively. In this particular example, 27.2% of the reported values fell outside of the ranges in both cases. However, in most instances the figures in the two columns are not the same. As one would expect, more values fell outside of the ranges based on the stated values than those based on the mean. But the differences are not large, and the averages of the figures in the two columns are 47.6% and 40.9%, respectively.

Table 1 shows that total reducing substances were estimated most accurately, with inorganic phosphorus a close second. Nonprotein nitrogen and cholesterol proved to be the most difficult to determine correctly. The percentage of unacceptable values based on the mean ranged from 17.8 to 67.0%, indicating a need for a great deal of improvement.

In Table 2 is given the general distribution of the results according to their magnitude of error. The data show that many values were grossly inaccurate.

Figure 1 is a scatter diagram for total reducing substances prepared by plotting Sample A values against their corresponding (obtained by the same laboratory) Sample B values. The values used for plotting in the majority of cases were the averages from duplicate determinations. Figures 2–8 are similar diagrams for all of the other components except cholesterol. These charts show not only the actual values obtained for both specimens but also the correlation between the two sets of values. The vertical and horizontal lines, drawn to outline the acceptable ranges (based on stated values), divide the charts into nine

sections of areas. The number of dots or points in each area is printed on the charts. Each point represents a different laboratory.

The center section contains points plotted from values which were both acceptable, and the upper right-hand section, points plotted from

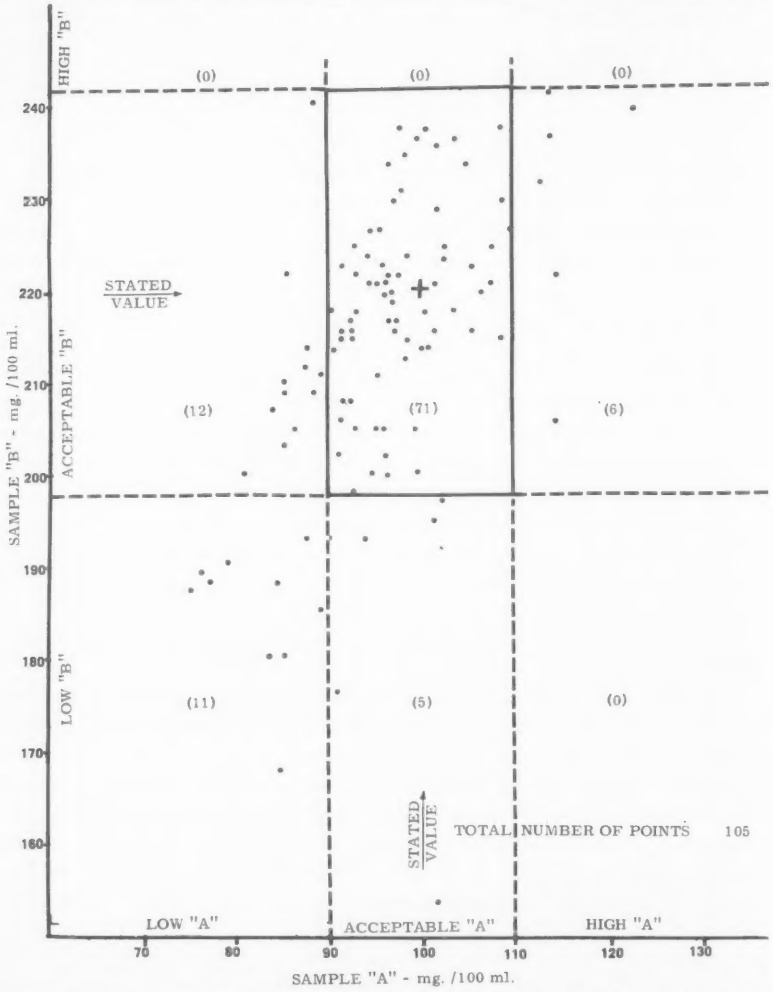


**Fig. 1.** Scatter diagram for total reducing substances. Plot of Sample A values versus Sample B values.

values which were both too high (that is, too high for Sample A and for Sample B). If errors had been due only to a lack of accuracy, say to improper standardization resulting in Values A and B both being either too high or too low, then all the points not in the central areas would have fallen either into the upper right (high A, high B) or lower left (low A, low B) sections. But 42.7% of all of the points on the

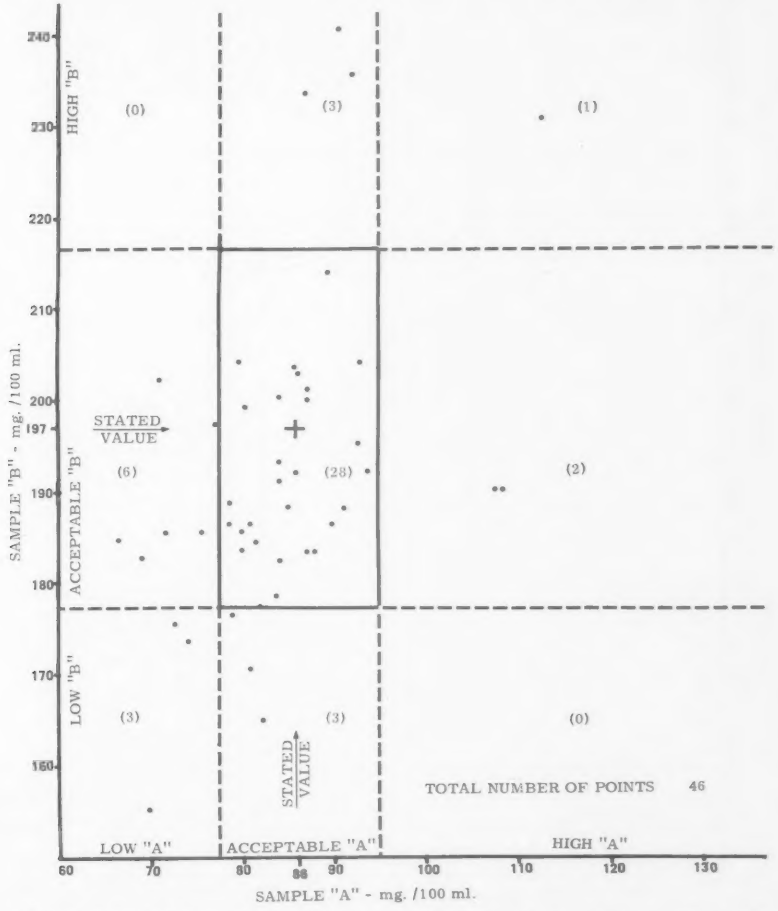


**Fig. 2.** Scatter diagram for "true" glucose. Plot of Sample A values versus Sample B values.

charts have fallen into the other areas. Therefore it is evident that there was a lack of precision as well as accuracy; and that poor technic, such as inaccurate pipetting, or the use of poor equipment, such as unmatched cuvettes, was responsible for many of the errors.

This conclusion is borne out by the data in Table 3, which compares

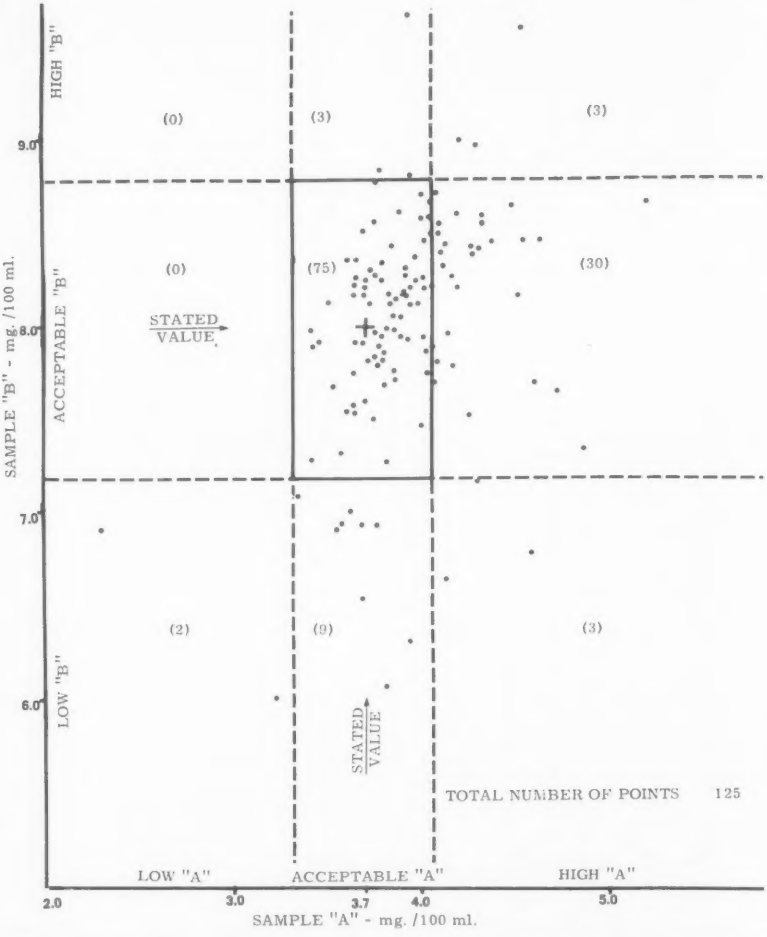


**Fig. 3.** Scatter diagram for inorganic phosphorus. Plot of Sample A values versus Sample B values.

the coefficients of variation of the reported values for Samples A and B with those of the ratios of the A and B values. The scatter of the ratios is obviously not very different from that of the reported values, again indicating that there was not only a serious lack of accuracy, but also of reproducibility or precision, in many of the laboratories.

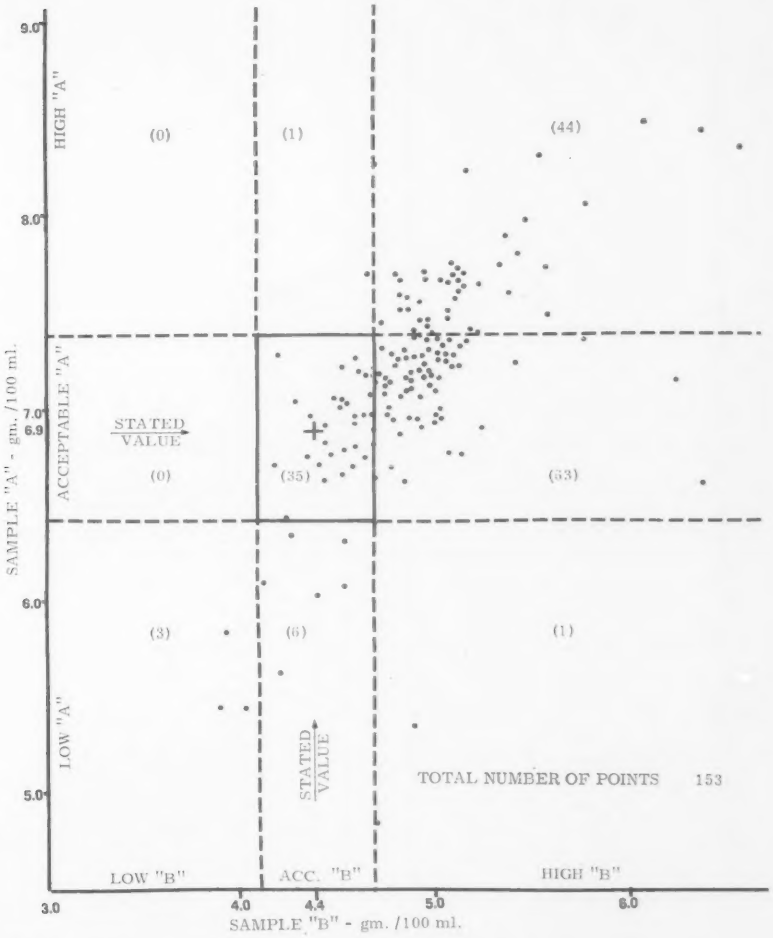


**Fig. 4.** Scatter diagram for total protein. Plot of Sample B values versus Sample A values.

Figures 9–12 are frequency charts prepared from the cholesterol values. They show the wide scatter of values reported for each of the four groups. The calculated mean values are in each case considerably higher than the absolute value of cholesterol in the specimen and are also considerably higher than the values estimated by the author for

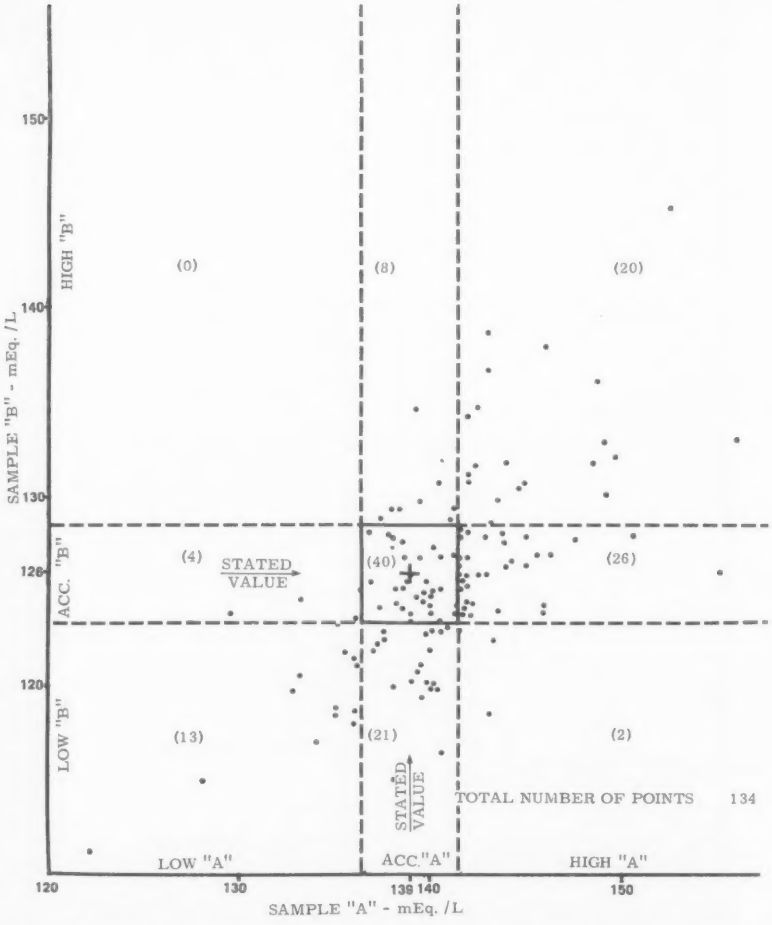


**Fig. 5.** Scatter diagram for sodium. Plot of Sample A values versus Sample B values.

the four groups. It is obvious that most of the cholesterol methods used lacked specificity.

Although it was not the purpose of this study to obtain information about the various methods used, it is worthwhile reporting that only 180 of the glucose values were obtained by true glucose methods. A large proportion of the other 469 values were estimated by a Folin-Wu

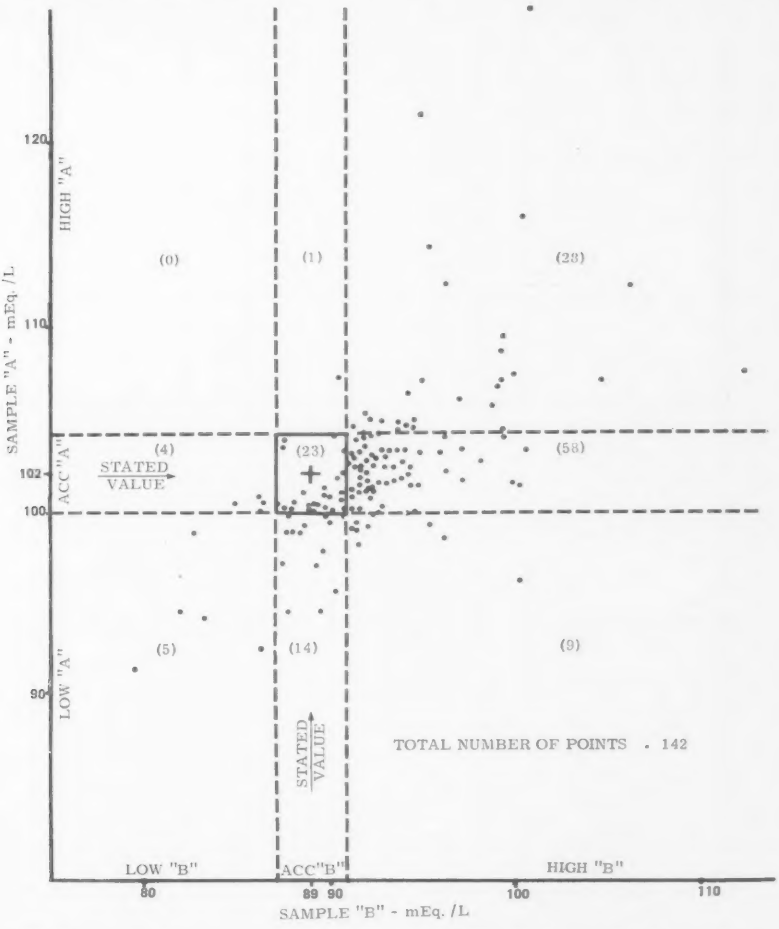


**Fig. 6.** Scatter diagram for chloride. Plot of Sample B values versus Sample A values.

technic. Almost all of the inorganic phosphorus values were obtained by the Fiske-SubbaRow method, and a majority of the laboratories used a biuret method for the determination of protein. Many different methods were used for cholesterol, with 137 of the 311 values being obtained by the Bloor method or some modification of this method, and 97 by a direct Liebermann-Burchard technic such as the Pearson,

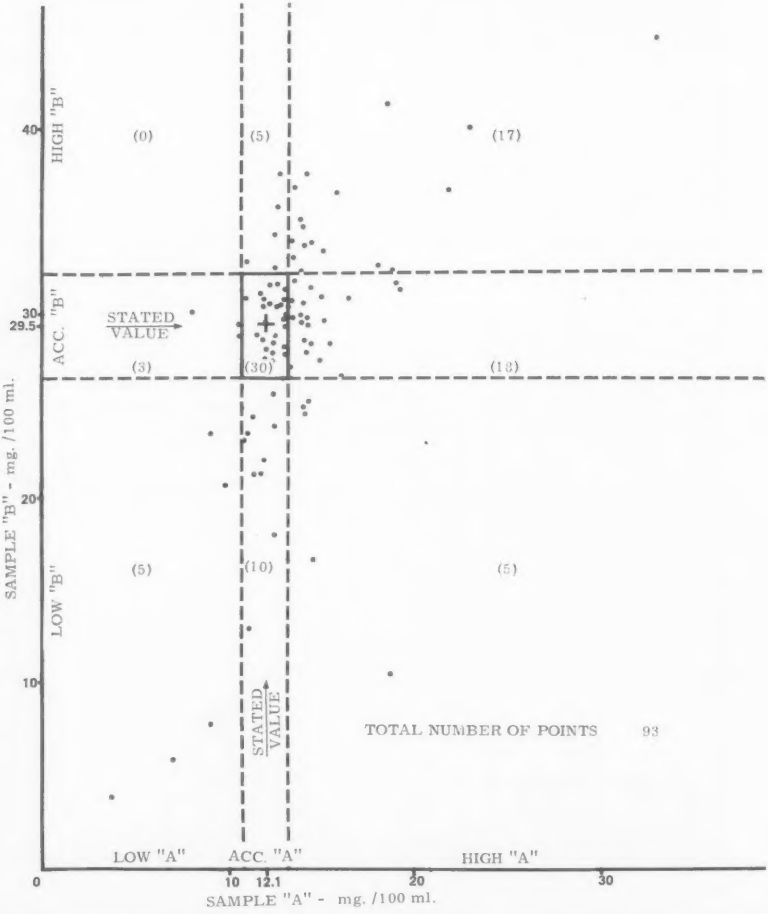


**Fig. 7.** Scatter diagram for urea nitrogen. Plot of Sample A values versus Sample B values.

Stern, and McGavack method. In the latter group were included the new "kit" methods which have become commercially available and which were used by eight laboratories. In most cases the results obtained by these methods were poor. Relatively few laboratories used

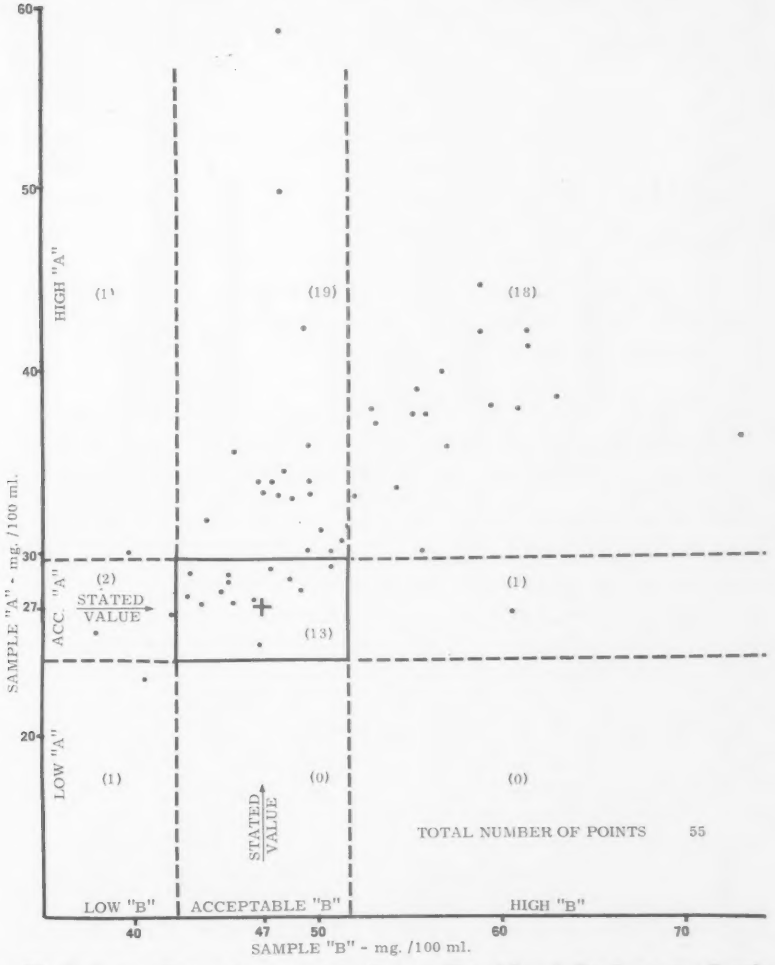


**Fig. 8.** Scatter diagram for nonprotein nitrogen. Plot of Sample B values versus Sample A values.

**Table 3.** Comparison of the Scatter of the Results for Samples A and B with the Scatter of the Ratio A/B,* as Expressed by Coefficients of Variation

| | *Coefficients of variation†* | | |
|---|---|---|---|
| *Component* | *Sample A* | *Sample B* | *Ratio* |
| Total reducing substances | 9.2 | 8.9 | 10.1 |
| "True" glucose | 12.1 | 8.7 | 7.9 |
| Inorganic phosphorus | 16.1 | 14.2 | 12.2 |
| Total protein | 8.6 | 9.2 | 7.5 |
| Sodium | 4.2 | 4.4 | 5.1 |
| Chloride | 4.9 | 5.2 | 4.1 |
| Urea N | 31.6 | 23.1 | 22.7 |
| NPN | 22.5 | 17.6 | 14.3 |

*Or B/A, if this gave a value greater than 1.

† $\frac{\text{S.D.}}{\text{mean value}} \times 100\% = \text{Coeff. of variation.}$

the Schoenheimer-Sperry method or some modification of it, but this method gave the most accurate values. Ferric chloride methods ran a reasonably close second.

## Conclusion

The chief purpose of this study was to determine whether or not there was a definite need for improvement in the performance of Canadian clinical chemistry laboratories. Since 61.7% of the laboratories which were originally contacted completed the survey, the results obtained should be quite representative of this performance. Actually the results should be better than those normally reported by the participating laboratories for several reasons. Very likely most of the participants gave special attention to the survey specimens. Better performances are to be expected from laboratories which take part in surveys than from those that do not take part because of fear of criticism or lack of interest of the personnel. Only relatively large laboratories took part, and generally speaking, we would expect to obtain better performances from large laboratories than from small ones. Therefore, since over 40% of the values obtained in this study were unacceptable by standards which are not overly severe, *it is obvious that there is a definite need for improvement.* Perry in the Maryland surveys (*4*) classified a laboratory as performing satisfactorily only when 90% of the reported values fell within the allowable limits; this is not an unreasonably high standard for an analytical laboratory. Very few laboratories approached this standard in this survey. It

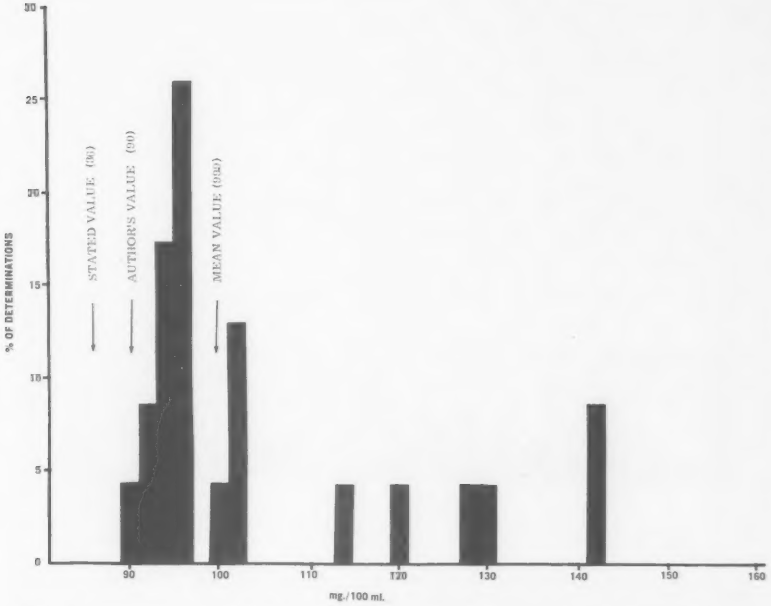


**Fig. 9.** Frequency chart of cholesterol values. Group 1, Schoenheimer-Sperry methods.

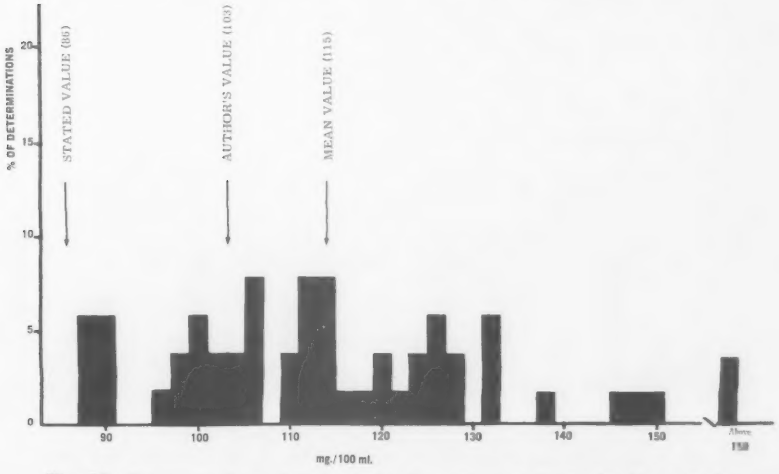


**Fig. 10.** Frequency chart of cholesterol values. Group 2, ferric chloride methods.

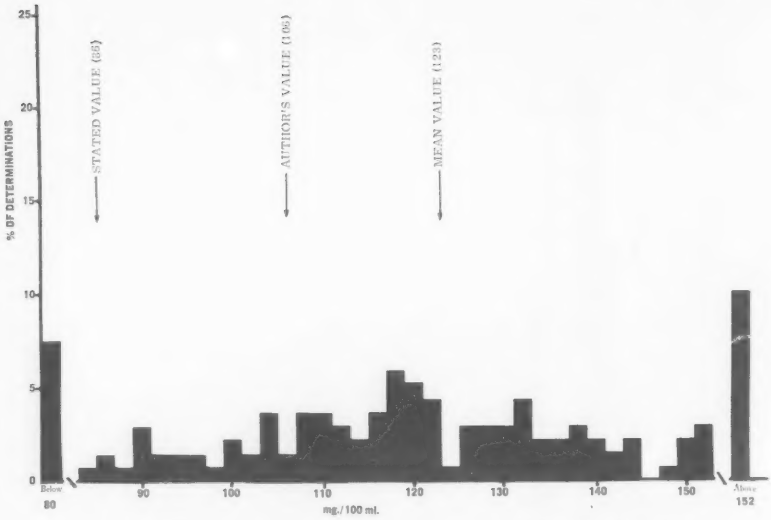


**Fig. 11.** Frequency chart of cholesterol values. Group 3, Bloor methods.

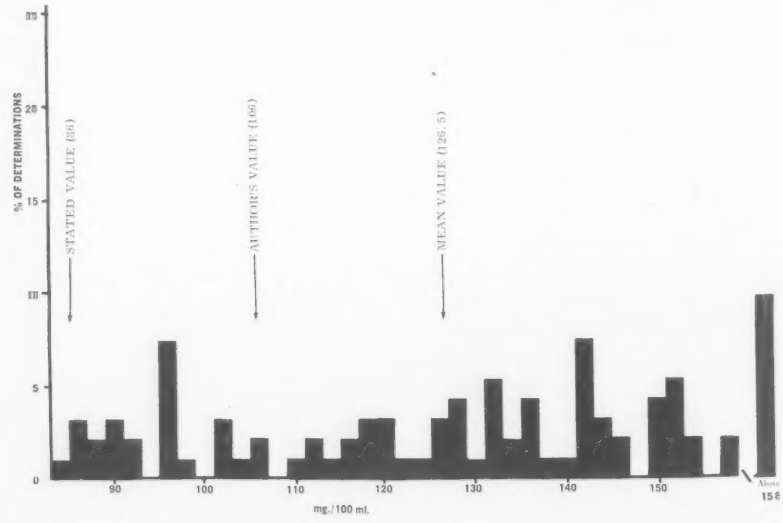


**Fig. 12.** Frequency chart of cholesterol values. Group 4, direct Liebermann-Burchard methods.

should be pointed out, however, that the results are no worse than those reported in similar surveys in other countries (*5*).

The second purpose of the study was to assist the individual participants, particularly those in the smaller hospitals and in isolated areas, to evaluate their own performances and to enable them to compare their results with those obtained by other laboratories. This purpose has been fulfilled, and it is known that the survey has exerted a considerable influence on the thinking of the participants regarding the accuracy of their analyses and the need for quality control. Also, many gross inaccuracies were detected, and it is certain that serious efforts were made to determine the cause of these errors and to eliminate them.

The data obtained have shown that there was a lack of both accuracy and precision in many laboratories. There is obviously a need for an improvement in the methods of detecting errors and of evaluating precision in the laboratory. An adequate quality-control system and the proper usage of control serums are indispensable for these purposes. It is also useful for every laboratory to take part in periodic studies such as this one in order to obtain an unbiased evaluation of its performance.

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# LETTERS TO THE EDITOR

*Dear Sir:*

Recently an *o*-toluidine method for blood glucose was reported in this Journal by Dubowski (*1, 2*). As the subject has been within our interest in the few last years we should like to present some comments on it.

A method for estimation of glucose in blood based on the color reaction of aldosaccharides with *o*-toluidine in glacial acetic acid was first published by Eric Hultman (*3*) in 1959. This method was adopted in our laboratory early in 1960. Since that time the method was also introduced in other clinical laboratories in Finland; i.e., by Mr. O. M. Forssell at the Joensuu Central Hospital. He then suggested the idea to Dr. Dubowski (*1*).

Hultman's method, however, was not satisfactory to us mainly because the results were frequently in error because of some unidentified impurities occasionally present in glacial acetic acid. A modified method, free of those interferences, using thiourea as a stabilizer in the reagent was then developed by us (*4*).

After the publication of Dukowski's paper we became interested to see if some differences from the Hultman procedure introduced by Dubowski could possibly eliminate the interferences. The original method of Hultman consists of the following steps: deproteinization of blood with 3% trichloroacetic acid, heating of 0.2 ml. of the supernatant with 3 ml. of the reagent in a boiling water bath for 8 min., cooling, and measuring the color at 625 m$\mu$. The reagent, 6% *o*-toluidine in glacial acetic acid, is prepared fresh before use.

The method published by Dubowski differs from the Hultman method in the following points: the reagent is aged for one week before use (then it should be stable for months), a greater portion of the filtrate (1 ml.) is used, heating time is 10 min., and readings are measured at 630-635 m$\mu$.

For testing we used some batches of glacial acetic acid which were earlier found by us (*4*) unsatisfactory in the Hultman method. These were BDH Analar, Riedel-de-Haen p.a., and a purum preparation. *o*-Toluidine was of puriss. p.a. grade of Fluka AG. The reagents made out of these chemicals were brown-violet with varying depths of colors, getting gradually darker on storage. They yielded reagent blanks equivalent up to 7 mg.% glucose in blood.

Determinations according to Dubowski were done by using a 50 mg.% glucose solution as the sample and a 100 mg.% glucose solution as the standard. Results showed that the great negative errors found in the Hultman method were not present in the Dubowski method. However, a definite positive error of 10–13% was detected. This indicates some nonlinearity when using these unsuitable batches of acetic acid. A new batch of Merck p.a. acetic acid gave a satisfactory result (51 mg./100 ml.).

It is to be noted that readings in the Dubowski method are much higher than those in the Hultman method (about three-fold). Interferences, if any, could

therefore be expected to occur at rather low concentration levels and possibly to be negligible in terms of mg./100 ml. of blood glucose.

We should like to point out that conditions at certain stages of the Dubowski method are not optimal. For instance the color is not fully developed in 10 min. Another important fact is that the color reaction is sensitive to water. As Hultman already stated, water content should not exceed 10%. Trichloroacetic acid inhibits the color production, making also the use of large volumes of filtrate unfavorable. Probably all these factors contribute more or less to the trend to nonlinearity as found when using the unsuitable batches of acetic acid in the Dubowski method.

*Malmi Hospital*
*Malmi-Helsinki*
*Finland*

AULIS HYVÄRINEN
ESKO A. NIKKILÄ

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the Clinical Chemist

## Certification Program in Toxicological Chemistry

The American Board of Clinical Chemistry is pleased to announce a program of Certification in toxicological chemistry. Scientists with adequate education and professional experience may now be certified in toxicological chemistry, without having to qualify for certification in the general field of clinical chemistry. The general Clinical Chemistry certification program in existence since 1950 also remains in force.

### Qualifications and Requirements

Requirements for certification in toxicological chemistry include the following:

A. Evidence of good moral character and of high ethical and professional standing

B. Educational background which includes:

1. An earned Doctor of Philosophy or equivalent doctorate degree in the natural sciences, or a Doctor of Medicine degree, from an accredited institution

2. Satisfactory completion of accredited courses in analytical, inorganic, organic, and physical chemistry; and adequate education in chemistry at the graduate school level

C. Professional experience in one or both of the following categories, or an appropriate combination:

1. At least 3 years' full-time responsible experience in the practice of human toxicological chemistry in the immediately preceding 10 years, in laboratories maintaining standards acceptable to the Board

2. Holding, for a period of 3 years or more, of professorial rank or its equivalent in toxicology, biological chemistry, chemistry, clinical chemistry, legal medicine, pharmacology, or equivalent field in an academic institution acceptable to the Board, provided such applicants have had at least 3 years' full-time equivalent satisfactory experience in human toxicological chemistry teaching, research or practice

D. Satisfactory passing of written and/or oral examinations

Applicants whose educational and experience qualifications are satisfactory to the Board, and who file their applications prior to June 30, 1965, may be certified in toxicological chemistry without examination at the discretion of the Board.

### Application Procedure

The Board is prepared to accept immediate applications for certification in toxicological chemistry. A charge of $3.00 is made for the application forms. Requests for additional information and application forms should be addressed to:

DR. KURT M. DUBOWSKI, Secretary-Treasurer
American Board of Clinical Chemistry,
University of Oklahoma School of Medicine,
Oklahoma City 4, Okla.

## News Section

### Third West European Symposium on Clinical Chemistry

The Third West European Symposium on Clinical Chemistry will deal with the subject of water and electrolyte metabolism. It is the third organized symposium in a continuing series. Papers are read by invited speakers only, but attendance at the symposium is open to anyone interested in clinical chemistry.

The dates of the symposium, which will be held in Amsterdam, are May 17–18, 1963. Further details can be obtained from DR. G. SMITS, Laboratorium voor Propedeutische Chemie, Nieuwe Amstelstraat 6, Amsterdam (C.), Netherlands.

An interesting preliminary program, already arranged, is as follows.

PROF. DR. H. H. USSING, Copenhagen: Active sodium transport at the cellular level.

PROF. DR. A. LEAF, Boston: The transepithelial transport of sodium.

DR. S. L. BONTING, Bethesda: A sodium and potassium dependent A.T.P.-ase.

DR. J. CRABBE, Louvain: Influence of aldosterone on active sodium transport.

DR. K. KRAMER, Gottingen: Active sodium transport in renal tubules.

DR. CH. TOUSSAINT, Brussels: Passive sodium transport in renal tubules.

DR. F. MOREL, Sacley: Influence of ADH on active sodium transport.

DR. I. H. MILLS, London: Extra adrenal regulation of sodium excretion.

DR. S. W. STANBURY, Manchester: Calcium and phosphorus metabolism in uremia.

DR. F. H. WOLTHUIS, Amsterdam: Evaluation of protein metabolism.

DR. F. PARSONS, Leeds: Dietary treatment and anabolic steroids in renal failure.

DR. J. H. HESS THAYSEN, Copenhagen: Anabolic steroids in renal failure.

DR. F. BOEN, Rotterdam: Treatment of chronic renal failure with dialysis.

## Section News

### Northeast Section

The first meeting of the season was held on Oct. 9, at which time DR. J. J. VITALE spoke on "Clinical and Experimental Nutrition Studies in Cali, Colombia, South America."

### Philadelphia Section

The section has held a series of meetings. In October the subject of discussion was "Biochemical Application of Infra-Red Spectroscopy," presented by DR. H. P. SCHWARZ. In November, DR. HAROLD PERSKY spoke on "Assessment of the Plasma Hydrocortisone Level in Man." In January, DR. M. A. SPIRTES discussed "The Mode of Action of Psychotherapeutic Drugs."

In addition, during December, the section jointly sponsored, with the American Association for the Advancement of Science, a symposium on "Newer Application of Instrumentation in Biological Analysis." The usefulness of gas chromatography in clinical chemistry was reviewed by D. A. TURNER; *p*H methodology, by S. R. GAMBINO; fluorescence assay, by S. UDENFRIEND; and recent advances in electrophoretic analysis, by S. RAYMOND.

The program of the section also included two Chemiclinics, both dealing with the subject of "Instrument Control." These were headed by DR. CARL ALPER.

### Cleveland Section

The section held its first meeting of 1963 on Feb. 5 at the Bunts Auditorium in the Cleveland Clinic. DR. JACK R. LEONARDS, Associate Professor of Biochemistry, Western Reserve University, School of Medicine, led the discussion on "Methods of Glucose Determination."

DR. HERBERT THOMPSON and his associates invited members of the section to Youngstown for the April meeting. This meeting will be held on Apr. 2. The Section will be dinner guests of the Youngstown group.

### American Society of Biological Chemists

DR. HARRY SOBOTKA, Chemist to Mount Sinai Hospital, New York, will head the newly formed Committee of Clinical Chemistry of the American Society of Biological Chemists.

### Lectures in Japan

DR. HARRY SOBOTKA, Chemist to Mount Sinai Hospital, New York, has been invited by the Chemical Society of Japan to lecture on holothurin at their annual meeting on Apr. 1 in Tokyo. During his stay in Japan he will lecture at the Universities of Tokyo, Kyoto, and Kyushu, and at the Institute for Protein Research of Osaka University. Dr. Sobotka will visit a number of clinical chemistry laboratories and discuss problems of common interest.

## National Treasurer's Report

### Balance Sheet as of June 30, 1962

ASSETS

| | |
|---|---|
| *Current Assets* | |
| Checking and savings accounts | $32,312.93 |
| Accounts receivable | 37.87 |
| Advances | 1,791.32 |
| Total current assets | $34,142.12 |
| *Fixed Assets* | |
| Office furniture and equipment | 1,080.55 |
| TOTAL ASSETS | $35,222.67 |

LIABILITIES

| | |
|---|---|
| Total current liabilities | $ 254.00 |
| Total net worth | 34,968.67 |
| TOTAL LIABILITIES AND NET WORTH | $35,222.67 |

## Van Slyke Symposium

The Metropolitan Microchemistry Society will hold a special Symposium in honor of DR. DONALD D. VAN SLYKE'S contribution to that specialty. The symposium will be held on Apr. 19, 1963, at the Hotel Manhattan, New York City. The program will be as follows.

ROBERT A. HOFSTADER, *General Chairman*

*Technical Session*

1:30–2:15 DR. V. DOLE (Rockefeller Institute): Analysis of Particulate Fat in Blood Plasma.

2:15–3:00 DR. P. KIRK (University of California): Some Instrumental Trends in Forensic Microanalysis.

3:00–3:30 Intermission—Coffee

3:30–5:00 Panel discussion: Applications of Van Slyke Apparatus

DR. A. STEYERMARK (Hoffman-LaRoche): In Chemical Analysis.

DR. J. SENDROY (U. S. Naval Medical Research Institute): In Medical Research.

DR. S. NATELSON (Roosevelt Hospital): Recent Advances in Clinical Analysis.

*Reception and Dinner*

| | |
|---|---|
| 5:30–6:15 | Cocktail hour |
| 6:30–8:00 | Dinner |
| 8:00 | Presentation to DR. DONALD D. VAN SLYKE |

Fees: Technical session, $2.00; reception and dinner, $8.00.

For further information please contact ROBERT A. HOFSTADER, Esso Research and Engineering Co., Linden, N. J.

### Comprehensive Laboratory Survey under Auspices of College of American Pathologists

Nationwide surveys held each year under the auspices of the Standards Committee, College of American Pathologists are a means of evaluating the present status of laboratory procedures. Participating hospitals benefit in that the survey is a means of checking the accuracy and precision of their procedures with laboratories throughout the country.

Growing interest in the survey program has prompted the Standards Committee to offer a Comprehensive Laboratory Survey in May, 1963. This survey is designed to evaluate procedures in immunohematology, bacteriology, parasitology, and chemistry. Specimens will be provided for the following studies: serum bilirubin, 2 samples; cholesterol, 2; sodium, potassium, carbon dioxide, urea; hemoglobin, 2; blood group and type, 2; blood smear evaluation; spinal fluid protein and chloride; stool culture; parasite identification.

All pathologists and laboratory directors are invited to participate. Inquiries may be addressed to: Comprehensive Survey, College of American Pathologists, Prudential Plaza, Chicago 1, Ill.

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### Van Slyke Award

At the Metropolitan New York Section Meeting on May 7, 1963, the Van Slyke Medal will be awarded to DR. LEONARD T. SKEGGS, whose topic will be "The Clinical Chemist and Automation."

# Quantitative Gravimetric Fractionation of Brain Tissue

*Warren M. Sperry*

**With the procedure which is described, brain tissue is separated into six fractions, the sum of whose weights equals the dry weight of the tissue. Three of these primary fractions are further separated into subfractions. Recovery is quantitative at each step. The procedure, as applied to rat brain, gave consistent results; duplicate analyses of the two halves of brains, divided as equally as possible along the anteroposterior line, agreed closely, and the variation from rat to rat in the percentages of the various fractions in dry tissue was small, as illustrated by the following results: main lipid fraction, 40.65 ± 1.03 (S.D.)%; protein residue, 44.45 ± 0.87%; water-soluble fraction from Folch purification, 5.94 ± 0.23%; crude "strandin" fraction, 1.25 ± 0.05%. The average percentage of dry in fresh tissue was 24.87 ± 0.41. The method includes procedures for the separation of a fraction, obtained by treatment with acidified chloroform-methanol, into four well-defined fractions, of which one is characterized by extreme surface activity in aqueous solution. A small amount of lipid is so firmly bound in brain tissue as to resist extraction during exhaustive treatment with chloroform-methanol (2:1), acidifed chloroform-methanol, and acetone. Significant radioactivity was present in most of the fractions from the brains of rats which had received a peritoneal injection of acetate-$C^{14}$ 5–15 months previously during the period of early development.**

A REMARKABLE metabolic stability of certain lipids in the adult brain has been reported by several investigators. Over 20 years ago, Waelsch *et al.* (*1*) found little or no labeling of the unsaponifiable lipids of the

From the Departments of Biochemistry, New York Psychiatric Institute and College of Physicians and Surgeons, Columbia University, New York.

This paper, in condensed form, was presented as the 1961 Ames Award Lecture before the Metropolitan-New York Section of the American Association of Clinical Chemists, March, 1962.

This investigation was supported by Grant 54 of the National Multiple Sclerosis Society.

The author is indebted to Dr. R. M. C. Dawson for suggesting the use of incubation in Step B of the procedure described; to Dr. Marjorie B. Lees for advice during the first part of the work, in which the modified Folch procedure was used; and to Dr. Herbert L. Meltzer for performing the injections of acetate-1-$C^{14}$ or acetate-1,2-$C^{14}$ described.

Received for publication Oct. 12, 1962.

brain in adult rats whose body fluids had been enriched with deuterium, whereas under the same conditions there was marked labeling in very young rats (*2, 3*). These results indicated that the ability of the brain to synthesize cholesterol is lost in adult life. This interpretation was supported by the finding by several investigators of little or no labeling of cholesterol (1) in the brains of adult rats after feeding deuterium-labeled acetate (*4*) or after intraperitoneal injection of acetate-1-$C^{14}$ (*5–7*); (2) in brain tissue from adult rats (*8*) or humans (*9*) after incubation in vitro with acetate-1, 2-$C^{14}$; or (3) in brains of adult cats after perfusion with octanoate-1-$C^{14}$ (*10*). On the other hand, small but significant incorporation of $C^{14}$ into cholesterol of adult rat brain occurred after injection of acetate-1-$C^{14}$ into the cisterna magna by McMillan *et al.* (*6*), or into the cerebrum by Nicholas and Thomas (*7*). In unpublished experiments, Meltzer and I obtained similar results after injection of acetate-1-$C^{14}$ into the subarachnoid space of adult rats by the technic of Lindberg and Ernster (*11*). The apparent discrepancy between these and the earlier results has not been explained, but the finding by McMillan *et al.* (*6*) of no decrease in the specific activity of brain cholesterol thus labeled up to 72 days after the injection suggests that the labeling resulted from synthesis of cholesterol in the process of growth, which continues throughout life in the rat.

Further important evidence for the metabolic stability of cholesterol and of other lipids in the adult brain has been supplied by Davison and his colleagues in a series of studies in which labeling was induced in the brain by administration of cholesterol-4-$C^{14}$ (*12–14*), DL-serine-3-$C^{14}$ (*15*), $P_i^{32}$ (*16–18*), or glycerol-1-$C^{14}$ (*18*) during the period of early development when growth of the brain and myelination are proceeding rapidly in chicks, rats, and rabbits. In all of these experiments the labels persisted in the brain lipids at relatively high levels well into adult life. Since the lipids thus shown to be metabolically stable, particularly cholesterol, cerebrosides, and sphingomyelin, are in all probability major components of myelin, Davison *et al.* (*15*) concluded: "The uniform persistence of these three myelin lipids over this long period is consistent with the belief that when once deposited in the myelin sheath at the time of its formation, they undergo little subsequent 'turnover'; the myelin sheath must, therefore, be regarded as one of the more permanent tissue elements."

This concept is of such manifest importance that it is worthy of further study, and more than 2 years ago I undertook an investigation similar to those of Davison and his colleagues but with a somewhat

different approach. Instead of aiming at the labeling of a particular lipid, the plan was to induce labeling of as many components of the brain as possible by administering acetate-$C^{14}$ to rats during early development and to determine the distribution of the isotope in the brain at various intervals thereafter. For this purpose, a procedure for quantitative, gravimetric fractionation of brain tissue was required. The description of such a procedure is the primary purpose of this communication.

## Method and Materials

### Special Apparatus

*Tissue Homogenizer* The apparatus* (Fig. 1 and 2) is similar to the tissue disintegrator which was previously described (*19, 20*), but it is made of Teflon except for the piston assembly, which is of stainless steel.

*Funnels with Fritted Discs Pyrex No. 37730F* Equipped with glass stoppers as shown in Fig. 3 and 4, these are calibrated as follows. The funnel is inverted and the space under (now above) the filter and the stem are filled with water. The stopper is inserted in the stem and the funnel is clamped in an upright position. Water (4 ml.) is added, and the height above the lower edge of the fritted disc is measured with a vernier caliper. Readings are taken at 1-ml. intervals to 8 ml. and plotted on coordinate paper.

*Centrifuge tubes, 40 ml., graduated, Pyrex No. 8144* Stoppers are ground as previously described (*19*) and attached with fine nichrome wire.

*Flasks, 50 ml., round-bottom, with standard taper 24/40 joints* These are equipped with nooses made of fine nichrome wire to permit hanging in a balance.

### Solvents and Solutions

All solvents, except chloroform, are distilled through a 40-plate Oldershaw column and tested from time to time for absence of residue. Chloroform is distilled through a short column from $K_2CO_3$ into sufficient ethanol to make the final concentration 1%.

*Chloroform-Methanol (2:1, v/v)* [later described as C-M]

*Chloroform-Methanol (1:2, v/v)*

*Ethanol, 50%*

*Buchler Instruments, Inc., Fort Lee, N. J.

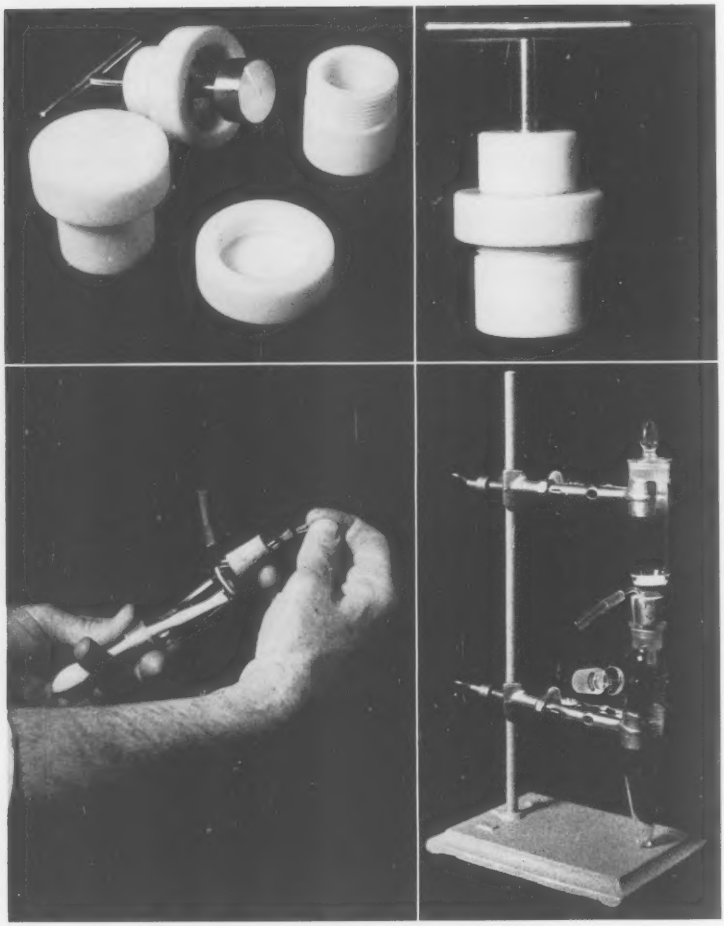

**Fig. 1.** Tissue homogenizer, disassembled. Dimensions of Teflon cups are: inside diameter, 2 cm.; inside height, 2.85 cm.; thickness of wall, 4 mm. Clearance between stainless steel piston and cylinder is 0.007 in. Height of piston is 9 mm. Bottom face of cylinder, upper face of cup wall, inner faces of head and caps, and top and bottom faces of piston are machined to smooth, plane surfaces which are perpendicular to vertical axis. **Fig. 2.** Tissue homogenizer, assembled. **Fig. 3.** Insertion of stopper in stem of funnel at Step A. Manner of holding the funnel and spatula during washing of space under filter is also shown. **Fig. 4.** Assembly of apparatus during incubation at Step B.

*Pure Solvents Upper Phase (PSUP) (21)* Methanol-water-chloroform (48:47:3, v/v/v).

*Buffered 0.5 M NaCl Solution in Upper Phase (22)* NaCl (6.084 gm.) and 0.583 gm. of Tris are dissolved in about 70 ml. of water and titrated with N HCl to *p*H 8.8. The solution is transferred quantitatively to a 100-ml. volumetric flask and made to volume; 94 ml. are mixed with 96 ml. of methanol and 6 ml. of chloroform.

*Acidified Solvent (23)* Chloroform-methanol concentrated HCl (20:10:0.1, v/v/v).

### General

Wherever applicable, previously described technics (*19, 20*) are used. Transfer apparatus (Fig. 2C in *Methods of Biochemical Analysis* (*19*)) is made with corks sprayed with Teflon aerosol (Fluro-Glide)* instead of rubber stoppers.

#### Protection Against Oxidative Degradation

Removal of solvents is carried out either by lyophilization, or in a rotary evaporator, or in a stream of pure nitrogen at about 45° (*19*). Without delay, dried samples are placed over Drierite† and paraffin in a vacuum desiccator which is evacuated with an efficient oil pump. Pure nitrogen is admitted and the desiccator is evacuated; this process is repeated once more. The extreme precautions described by Rouser *et al.* (*24*) (use of oxygen-free solvents, etc.) were not considered to be essential to the present study and were not applied, but they could be if desired. Rouser *et al.* (*25*) obtained essentially the same results whether or not extraction of lipids from beef brain was carried out in the absence of oxygen.

#### Weighing

Since the success of this procedure depends on the precision of weighing, the technic used is described in detail. Semimicro, Sartorius Selecta balances are used for all weighings. The Teflon cups and caps (Fig. 1) are washed carefully with water and methanol and thoroughly dried before weighing. Glass apparatus to be weighed is carefully cleaned, usually with sulfuric acid-chromate or by ultrasonic

*Scientific Glass Apparatus Co., Bloomfield, N. J.
†Fisher Scientific Co., New York, N. Y.

treatment. It is filled with tap water and emptied at least 3 times and rinsed with distilled water 3 times. After drying, the apparatus is rinsed 2 or 3 times with small amounts of C-M and clamped in an inverted position until dry. The outer walls of flasks and centrifuge tubes are rinsed with distilled water and wiped carefully with a clean, lint-free towel. Funnels are wiped first with a moist towel and then with a dry towel. After wiping, apparatus is handled only with an hemostat or tongs; it is kept *in vacuo* over Drierite and paraffin overnight. Small apparatus (5-ml. flasks and filter funnels) is equilibrated in Dri-Jar desiccators (*26*) for about 0.5 hr. before weighing. Large apparatus is transferred to the balance from the vacuum desiccator. The same timing and sequence are used for the tare and gross weighings. A stopwatch is started when the balance is released, and the weight is read at a time determined by the characteristics of the balance (30-35 sec. with the balances used in this work). The zero point is read after each weighing at the same time interval, and the weight is corrected by one-half of any deviation. With every one to four pieces of apparatus, one of the same type and size is weighed as a control, and the net weights are corrected for any change in this weight. With small apparatus such as 5-ml. flasks this correction is small, usually $<0.05$ mg., rarely $>0.1$ mg., but with large apparatus such as 50-ml. flasks it may be quite large, 0.5 mg. or, rarely, even more. In some instances this correction has been larger than the net weight. The inclusion of control weights is essential to the achievement of the precision shown under *Results and Discussion*. It is also essential with large apparatus for the following reason: In weighing small apparatus, the balance comes to rest in about 0.5 min. and does not change for several minutes, but with large apparatus, after apparent rest is reached at 30-35 sec., a slow drift in the direction of a smaller weight begins and continues for 4–6 min. It has been impossible to relate this phenomenon to the presence of moisture or to electric charge. To wait 4–6 min. for the balance to come to rest would require an inordinate amount of time in weighing, and would increase the risk of oxidation through exposure of samples to air. In a large series of weighings it was noted that the amount of drift was nearly the same for all of the members of a set, including the control. This makes it possible to record the weights at 30–35 sec. and to apply the control correction. Weights thus obtained agreed very closely with those obtained in the same flasks by waiting for the balance to come to rest.

## Procedure (Fig. 5)

### Step A

Funnels and centrifuge tubes are tared and assembled as shown in Fig. 6. The tissue to be analyzed (one-half of a rat brain in the present work) is placed as rapidly as possible after killing the animal in a tared Teflon cup, the cover is screwed on tightly, and the cup is weighed. Methanol (4 ml.) is added, and the homogenizer head (Fig. 1) with the piston at its uppermost position is screwed on and tightened with two large pliers. The piston is forced as far down as it will go, twisted slightly, and pulled to the top, again with a twist to force tissue from between the surfaces of the piston and the Teflon head. This process is repeated briskly for about 35 complete strokes, an arbitrary number found to give good homogenization. At the last stroke, the piston is pulled tightly against the head, and the apparatus is hung by the handle in a spring clamp. The cup is unscrewed with care to keep it in a

**Fig. 5.** Diagram of procedure. Steps are lettered and fractions are numbered arbitrarily in order in which they are described in text.

vertical position, and the homogenate is transferred to the funnel with gentle suction, as shown in Fig. 6. Immediately after the transfer is finished, the suction is relieved by opening a spring clamp on a large, rubber, side tube. If there is delay at this point, tissue will dry on the inside of the transfer tube; such dried tissue cannot be removed. A 1-ml. beaker is held under Tube S (Fig. 2C (*19*)) and the outside is rinsed with two or three drops of methanol. This rinse is sucked over by closing the rubber side tube momentarily with the fingers. The piston assembly is removed from the clamp, and the piston is pushed down about ¾ in. and held at an angle over the cup. With a transfer pipet (Fig. 2A (*19*)) about 0.5 ml. of methanol is run over the piston to wash some, but not necessarily all, of the tissue which usually adheres to it into the cup. The head is screwed on as before and the piston is operated for five strokes. The homogenate is transferred as before. This washing procedure is repeated 3 more times. After the last wash the inner end of Tube S is rinsed with two or three drops of methanol, and a little methanol is sucked over from the 1-ml. beaker. Usually, a few

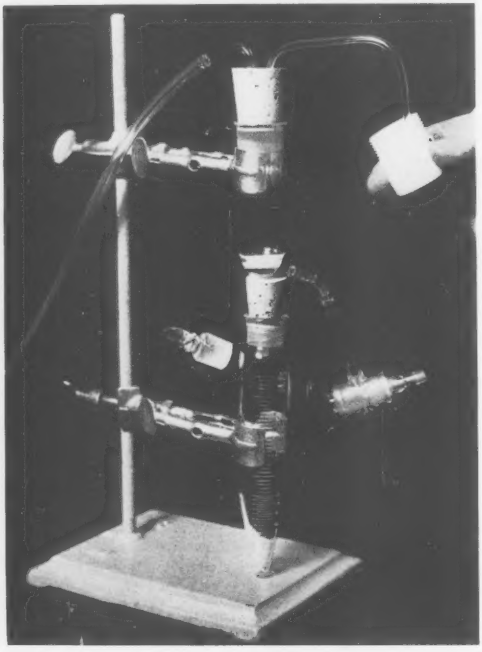

**Fig. 6.** Apparatus assembled for Step A. Procedure for transfer of homogenate from Teflon cup to funnel is shown. In this and Fig. 3, 4, and 7, apparatus was not photographed in actual use because of technical difficulties.

particles of tissue remain on the Teflon head and piston, and occasionally in the cup, and there is a little dried tissue on the outside of the transfer tube. After the chloroform has been added (see below), this tissue is transferred to the funnel with the aid of a small metal spatula.

Without delay, the height of liquid in the funnel above the lower edge of the fritted disc is measured with a vernier caliper, and the volume is determined from the calibration chart. After subtraction of the calculated volume of water from the tissue (wt. in grams $\times$ 0.75), twice the volume of chloroform is added. Stirring is not necessary. The funnel is loosely stoppered, and filtration is allowed to proceed by gravity for about 3 hr., after which time the major part has usually filtered.

Filtration of the remaining suspension is accelerated by the application of gentle suction, which is continued until most of the solution has filtered; the suction is relieved before the cake of tissue has been sucked dry. The wall of the funnel is washed with about 0.8 ml. of C-M, added with a transfer pipet. The tissue is stirred into uniform suspension with a small porcelain spatula. Suction is applied until most of the solution has filtered and the cake appears to be moist. This washing procedure is applied 9 more times. If necessary, at about every third wash, the solvent is ejected onto the spatula to free it from large masses of tissue suspension. After the tissue has been stirred into suspension at the tenth wash, the spatula is freed of adhering tissue by forceful washing with a little more C-M.

The filter assembly is removed from the centrifuge tube, the spatula is raised a little, it and the funnel are grasped with one hand, and the assembly is tipped back far enough (Fig. 3) to permit a small amount of C-M to be injected into the stem with a fine-tipped pipet. The space under (now above) the filter is rinsed by rotating the assembly. The filter is tipped up so the C-M will run into the centrifuge tube. The outside of the stem is rinsed with a little C-M. This washing procedure is repeated twice more. After the last wash, the space above the filter is filled with C-M, the stopper is tightly inserted in the stem (Fig. 3), the outside of the stem and stopper are rinsed with C-M, and the assembly is transferred to another centrifuge tube.

#### Step B

About 4 ml. of acidified solvent are added to the funnel and the tissue is stirred into a uniform suspension with care to break up all lumps. Pigment is extracted from the tissue, which becomes almost pure white. The spatula is lifted to the mouth of the funnel, and any tissue

adhering to it is scraped loose with a small metal spatula. Both spatulas are rinsed with acidified solvent. This process is repeated until no white residue is left on either spatula. About 2 ml. of acidified solvent, applied in 4 or 5 portions with a small transfer pipet, are used in washing. The assembly (Fig. 4) is placed in an incubator at about 38°, and, after equilibration for 0.5 hr., the funnel is stoppered and left over night.

The assembly is removed from the incubator, the upper stopper is removed, and the funnel is raised in the clamp so the lower stopper is just above the mouth of the centrifuge tube. The stopper is grasped with a small hemostat which has been rinsed with C-M. The funnel is rotated gently in the clamp until the stopper is loosened. While still over the centrifuge tube, it is rinsed with a little C-M. The funnel is lowered, the cork is tightened, and gentle suction is applied. Filtration and washing are carried out as in Step A except that only six washes with C-M are applied. The space under the fritted disc is rinsed as in Step A.

### Step C

The funnel is transferred to a tared 25-ml. suction flask (Fig. 7), about 3 ml. of acetone are added, and the tissue is stirred into suspen-

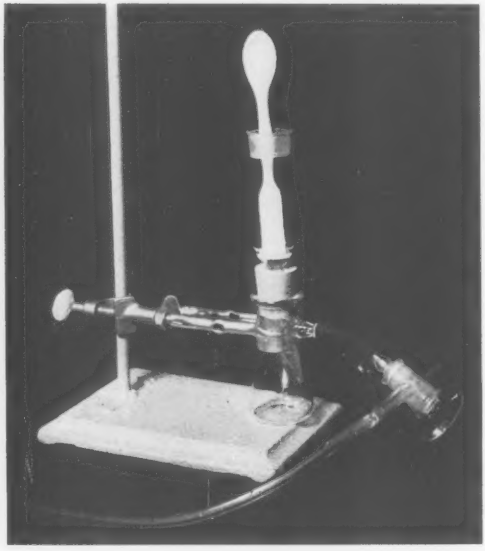

**Fig. 7.** Assembly of apparatus during washing with acetone at Step C.

sion. The solvent is filtered, and the tissue is washed 5 times with 2-ml. portions of acetone, the tissue being stirred into suspension with the suction off at each wash. Before the last wash is filtered, the spatula is freed of adhering tissue as described in Step A.

The acetone is evaporated from the filtrate with a stream of nitrogen (*19*), and the residue is weighed (Fraction 1). Acetone is evaporated from the residue in the funnel with a gentle stream of nitrogen, and the residue is weighed (Fraction 2).

#### Step D

C-M is added to the filtrate from Step A to the 30-ml. mark. Water (7.5 ml. minus the weight of tissue, in grams) is added, the stopper is moistened with water and inserted tightly, and the contents are equilibrated by slow inversion and reversion 30 times and allowed to stand over night. The tube is centrifuged 5 min. at <1500 rpm. As much as possible of the upper phase is sucked with a Van Slyke-Rieben (*26*) pipet into a tared 50-ml. flask. This is best done by clamping the tube on the rod of a Lab-jack and raising it until the horizontal tip of the pipet is about 2 mm. above the interface. The suction should be just sufficient to start siphoning. Good illumination should be used and care should be taken to avoid any transfer of lower phase. Five milliliters of PSUP are added slowly down the wall of the tube which is rotated so the entire wall is washed. There should be no mixing with the lower phase. The upper phase is stirred gently with a small metal spatula to mix the residual upper phase with the PSUP. The spatula is rinsed with 2-3 drops of PSUP, and the upper phase is sucked off as before. This washing procedure is repeated 4 more times.

The upper phase is concentrated with a stream of nitrogen to about 10 ml., lyophilized, and weighed (Fraction 3).

#### Step E

Twenty milliliters of NaCl solution in buffered PSUP are added to the lower phase from Step D, the phases are equilibrated, the tube is allowed to stand over night and centrifuged, the upper phase is removed, and six washes with PSUP are applied, all as described in Step D. The upper phase is discarded.

#### Step F

Precipitate in the form of a rubbery "skin" should appear at the interface in Step E. With a fine wire this is teased and pulled up onto the wall. The clear solution is sucked into a tared 50-ml. flask. As the

surface of the liquid is lowered, any precipitate which may have remained at the interface adheres to the wall. The tube is washed 5 times with small portions of C-M (*19*). The precipitate is not dislodged by the solvent which runs down the wall during washing. The centrifuge tube is dried and weighed (Fraction 4).

The lower phase is evaporated at <45° with a stream of nitrogen (*19*). When the volume reaches 2 or 3 ml., evaporation becomes very slow. A film of lipid forms over liquid which is largely aqueous. At this point about 5 ml. of acetone are added and the flask is swirled to form a milky emulsion. Evaporation is continued until most of the acetone is gone. This process is repeated with smaller amounts of acetone until the residue appears to be dry. Small amounts of acetone are added to the dry-appearing residue and evaporated twice more. The residue is weighed (Fraction 5).

Step G

Immediately after weighing Fraction 5, about 1 ml. of C-M is added, and the flask is stoppered. A precipitate, presumably of proteolipid protein (PLP), remains undissolved. The suspension is transferred with a small transfer pipet (Fig. 2A (*19*)) drawn to a fine tip to a tared filter funnel (Pyrex No. 36290M), which is arranged to feed into a tared 5-ml. volumetric flask by means of a 2-holed stopper and a short piece of rubber tubing. The other hole of the stopper carries a glass tube through which suction may be applied. The 50-ml. flask is washed 5 times with small portions of C-M. No attempt is made to transfer all of the precipitate to the funnel. The bulb is removed from the transfer pipet, which is rinsed 3 times with small amounts of C-M. After the transfer is complete, suction just sufficient to induce slow filtration is applied. There is danger that the filter may clog if the suction is too great. If this should happen, the sample may be saved by scraping the precipitate from the filter with a small metal spatula. The funnel is washed 5 times with small portions of C-M.

The funnel and the 50-ml. flask are dried and weighed (Fractions 6 and 7, respectively). The solution in the 5-ml. flask is taken to dryness with a stream of nitrogen and with the aid of additions of acetone as dryness is approached. After weighing, Fraction 8, which is the main lipid fraction, should dissolve completely in C-M.

Step H

Fraction 3, the residue from the upper phase from Step D, is dissolved in a minimal amount of water and transferred with a small

transfer pipet and five washes to a dialysis sack prepared as follows. A length of wet 15/16 in. (flat width) Visking tubing is passed onto a piece of 15-mm. glass tubing, about 5 cm. long, constricted at the lower end to about 10 mm., flared at the upper end, and equipped with a long piece of fine nichrome wire. The Visking tube is secured to the glass tube with a rubber band and securely tied with a double knot to form a sack which extends 4-5 cm. below the glass support. The excess tubing is cut off at the knot. The sack is filled with water, suspended by means of the nichrome wire in water in a large flask, and boiled for several hours. Just before use, the sack is hung by means of the glass tube in a spring clamp and washed several times by filling with water and sucking the water out. The outside is also copiously washed. In transferring the sample, care is taken to pass the tip of the pipet well into the sack before the solution is expelled. The bulb is removed, and the pipet is rinsed with water.

About 280 ml, of water are placed in a thoroughly cleaned 600-ml. Erlenmeyer flask. The sack is lowered into the flask until the surface of the solution in it is at a level with the surface of the water and secured in that position by means of the nichrome wire and a cork protected with aluminum foil. Dialysis is allowed to proceed at 4° for 2 or 3 days.

The sack is suspended in a spring clamp, and the solution is transferred by suction to a tared 50-ml. flask. Washing is carried out with a polyethylene wash bottle whose flexible nozzle is inserted through the glass support tube and turned so the entire inner surface of the sack is rinsed with a small amount of water which is sucked into the flask. This is repeated several times. The solution is lyophilized and the residue is weighed (Fraction 9).

The dialysate is transferred to a 1-L., round-bottomed flask which has been thoroughly washed after standing at least 24 hr. full of $H_2SO_4$-chromate. The solution is concentrated with a rotary evaporator to about 1 ml. and transferred by suction to a tared 5-ml. volumetric flask. Washing is carried out as previously described (*19*) with 50% ethanol. At the first wash, the flask is turned so the solvent (about 1 ml.) wets the entire surface. If the flask has been well cleaned, this is easily accomplished, and the following four washes run smoothly down the wall of the flask in a continuous film as the flask is rotated. With care, the total volume can be kept within 4 ml. Occasionally, some concentration of the solution in the 5-ml. flask may be necessary before the

washing is completed. The solvent is removed with a stream of nitrogen and the residue is weighed (Fraction 10).

Step I

The filtrate from Step B is transferred to a tared 50-ml. flask (*19*), concentrated with nitrogen at <40° to about 6 ml. (the original volume of acidified solvent), and lyophilized as follows. After being attached to the apparatus, the flask is partly immersed in a Dry Ice-ethanol bath in a beaker which is supported on a Lab-jack. After 5–10 min., the pump is started, and the bath is lowered when the pressure has been reduced to 2 mm. or less. The residue is weighed (Fraction 11).

Step J

Immediately after weighing, 2 ml. of C-M(1:2) are added to Fraction 11, a stopper is inserted, and the flask is allowed to stand for at least 2 hr. The suspension is transferred with the technic described in Step G to a tared 12-ml. centrifuge tube and centrifuged 5 min. at 1500 rpm. The clear, colored, supernatant solution is transferred with the pipet used in the previous transfer to a tared 5-ml. volumetric flask. A small amount of C-M (1:2) is added, the precipitate is stirred into suspension, and the centrifuging and transfer are repeated. This washing procedure is repeated 4 more times. The solution is concentrated in the flask with nitrogen to make room, if necessary. Drying is completed with nitrogen (*19*), and the residue is weighed (Fraction 12). The flask and centrifuge tube are dried and weighed to give Fractions 13 and 14, respectively.

Step K

Chloroform (1-2 ml.) is added to Fraction 12 and the flask is stoppered and allowed to stand 1-2 hr. The clear, highly colored solution is transferred with 5 washes with chloroform to a tared 5-ml. flask with the technic described at Step G. The solvent is removed with nitrogen and the residue is weighed (Fraction 15). The chloroform-insoluble residue is dried and weighed (Fraction 16).

Step L

Water (about 1 ml.) is added to Fraction 13 (in the 50-ml. flask) and the flask is stoppered and placed in the refrigerator until Step K has been completed. The water, which usually remains clear, is transferred with 5 small washes to the centrifuge tube. (All transfers in this Step are carried out with the technic described at Step G.) Water (about 1 ml.) is added to Fraction 16, most of which dissolves. This solution (or

suspension) is also transferred with 5 washes to the centrifuge tube. The contents of the centrifuge tube are stirred with a small metal spatula to suspend Fraction 14. The tube is centrifuged and the clear supernatant solution is transferred to a tared 50-ml. flask. A small amount of water is added to the centrifuge tube, the residue is stirred into suspension, the tube is centrifuged, and the supernatant solution is transferred to the 50-ml. flask. This washing procedure is repeated 4 times more. The solution in the 50-ml. flask is lyophilized and the residue is weighed (Fraction 17). The residues in the 5-ml. flask, 50-ml. flask, and centrifuge tube are dried and weighed to give Fractions 18, 19, and 20 respectively.

#### Step M

The water-soluble Fraction 17, after weighing, is dialyzed, and the nondialyzable fraction and dialysate are treated as described at Step H and weighed (Fractions 21 and 22, respectively).

### Notes on Procedure

#### Step A

The few particles of tissue which usually resist homogenization and transfer appear to be connective tissue. The amount appears to be very small, and its loss would have little effect on the results except possibly on dry weight.

During the time required for the transfer of the homogenate and washes, no filtration has been observed. Liquid does penetrate the fritted disc but the amount is judged to be too small seriously to affect the measurement of volume. That the desired ratio of chloroform to methanol (2:1) is achieved is shown at Step D. The volume of lower phase has always been between 21 and 22 ml. (in most experiments, between 21.5 and 21.9 ml.), as expected (*21*).

The suspension is allowed to filter by gravity for about 3 hr. for two reasons: (1) If suction is applied at once, there is some danger that the filter will be clogged; (2) it is possible, but not proved, that extraction of lipids is improved by the longer contact between tissue and solvent.

The exhaustive washing procedure was adopted on the basis of an extensive series of experiments in which the amounts of material removed by various sizes and numbers of washes were measured. Even after 10 washes, as described, very small amounts of weighable material are extracted by further washing.

The washing of the space under the filter is applied to guard against loss due to channeling during filtration. The moist tissue adheres

quite firmly to the filter and there is little danger of loss if the funnel is not tipped back too far.

Step B

The amount of tissue adhering to the spatula is minimized by the rinse at the tenth wash in Step A.

Leakage into the centrifuge tube during incubation has not exceeded 0.5 ml.

Step C

The treatment with acetone is carried out primarily to facilitate drying of the protein residue. Surprisingly, after the exhaustive treatment with solvents in Steps A and B, acetone always extracts weighable material, but the amount is small and it has not been further investigated.

Step D

The amount of water to be added is calculated according to Folch *et al.* (*21*).

Excessive agitation during equilibration may result in emulsions which are difficult to break.

The centrifuge should be at ambient temperature; if it has been heated by previous use the phases may become cloudy on standing at room temperature. There is usually a thin film of material (emulsion or fluff ?) at the interface after centrifuging. The volume of the lower phase should be 21–22 ml.

Step E

There should be no change in the volume of the lower phase during the treatment with the NaCl solution.

In two experiments, the upper phase and washes were taken to dryness with a rotary evaporator, and the residue was dissolved in a small amount of water and dialyzed. No weighable residue (after correction for the blank) remained in the dialysis sack. This result shows that there is no loss of nondialyzable material from the tissue, and the upper phase from the NaCl treatment is, therefore, discarded. It may be assumed that all dialyzable material was removed at Step D.

Step F

In some experiments, sufficient methanol (about 3 ml.) was added to cause the small amount of residual upper phase to blend with the lower phase. This probably is not necessary since a two-phase system forms as the volume is decreased.

Attempts were made to evaporate the lower phase with a rotary evaporator, with the Rotary Evapo-Mix,* or by lyophilization. All failed because of excessive foaming or explosive boiling as dryness was approached.

The treatment with acetone is applied primarily to facilitate drying, but it may increase the release of PLP (see below).

### Step G

There is a large, fortuitous variation in the relative amounts of precipitate which go into suspension (Fraction 6) and adhere to the flask wall (Fraction 17).

The transfer, filtration, and washing can easily be accomplished with a total volume of 3–4 ml. of C-M with the technic previously described by the author (*19*).

It is difficult to remove C-M completely from concentrated solutions of lipids. (Fraction 8 from one-half a rat brain contains about 100 mg.) The addition of acetone facilitates drying of such solutions.

### Step H

During the development of the procedure, a large number of blank dialyses was carried out. Despite all attempts to insure cleanliness, including excessive soaking and washing of the dialysis sacks, small but definite residues were always found, the amount in the small volume of water in the sack being almost as large as that in the large volume of "dialyzate." Boiling the sacks considerably reduced the blank, but weighable residues were still present. For the greatest precision, especially when the amounts of material being dialyzed are small, it is desirable, therefore, that blank dialyses be carried out and that corrections for the residues found be applied.

Fraction 9, largely strandin, should appear as a white, fluffy residue, a small weight of which occupies a large volume.

### Step I

In all of the experiments so far carried out, the filtrate from Step B was transferred to a 25-ml. volumetric flask, and, after dilution to volume and mixing, 5 ml. were pipetted for analyses. The pipet was rinsed into the centrifuge tube and the remaining 4/5 of the solution were transferred to a tared 50-ml. flask as described. The use of a centrifuge tube to receive the filtrate was continued from earlier experiments, where a Folch purification, similar to the procedure described in Step

*Buehler Instruments, Inc.

D, was applied. With the procedure as described, it might be better to filter directly into a 50-ml. tared flask.

In some experiments, C-M (1:2) was added to the dry residue, and the lyophilization was repeated to insure removal of HCl. No effect on the results was observed, and this procedure is probably unnecessary.

#### Step J

With the technic described, the solvent leaches material, including most, or all, of the reddish pigment, from a gelatinous residue which adheres to the wall of the flask. Little of the insoluble material is transferred to the centrifuge tube. In some experiments, the residue was scraped into suspension with a bent metal spatula so a large part of the insoluble material was transferred. No effect on the results was observed.

#### Step K

No precipitate has gone into suspension in chloroform under the described conditions in any of the experiments which have been carried out. The clear, highly colored solution is easily separated from the insoluble material.

#### Step L

The purpose at this step is to combine in one fraction all the water-soluble material extracted by the acidified solvent. Most of it is in Fraction 16, and it is not certain that any remains in Fractions 13 and 14; but to be sure, treatment with water is carried out as described.

#### Step M

Fraction 17 is exceedingly surface active, and care must be taken to avoid excessive foaming in the transfer to the dialysis sacks. Fraction 21, during lyophilization, should appear much as strandin does, i.e., as a fluffy material which occupies a large volume in relation to its weight. It is highly aquaphilic and surface active.

## Results and Discussion

In each of the 36 experiments which have been carried out in the development of the procedure, a rat brain was divided along the antero-posterior mid-line into two parts, as nearly equal as possible, and these were analyzed side by side with two objectives: (1) When the same technic was applied to both, the results gave a measure of the re-

producibility of the procedure. This is illustrated by some of the data from a typical experiment (Table 1). (2) The effects of changes in technic, applied to one of the half brains, were tested. This is illustrated by the study of procedures for isolation of PLP (see below).

### Dry Weight

In evaluating the procedure, particular attention was paid to the percentage of dry weight in fresh tissue since it is calculated from the sum of several weights (primary fractions) and would be expected, therefore, to reflect errors and inaccuracies. The results, as typified in Table 1, were satisfactory, especially so, in view of the expectation that there would be some differences between the results obtained on the two halves of a brain due to failures to divide the brain into exactly equal parts. An appreciable difference in the proportions of white and grey matter would have a considerable effect on the percentage of dry weight. In seven experiments carried out with the procedure essentially as described above, except for the omission of Steps E and F in the analysis of one half-brain (H-2) (see below for explanation), the average dry weight percentages for H-1 and H-2 were 24.86 and 24.88, respectively. The maximal difference between the two values was 1.03%, and the average difference was 0.34%.

**Table 1.** PRIMARY* FRACTIONS FROM A RAT BRAIN (EXPERIMENT 35)

| Fraction | | Weights (mg.) | | Percentages | | | |
|---|---|---|---|---|---|---|---|
| | | | | In fresh tissue§ | | In dry tissue | |
| No.† | Nature | H-1‡ | H-2‡ | H-1 | H-2 | H-1 | H-2 |
| 1 | Acetone extract | 0.32 | 0.27 | 0.03 | 0.03 | 0.13 | 0.11 |
| 2 | Protein residue | 109.93 | 104.86 | 11.13 | 11.10 | 43.90 | 43.54 |
| 3 | Water-soluble‖ | 14.25 | 13.45 | 1.44 | 1.42 | 5.69 | 5.59 |
| 4 | PLP¶ | 2.12 | —** | 0.21 | — | 0.85 | — |
| 5 | Main lipid + PLP | 106.76 | 104.50 | 10.81 | 11.06 | 42.63 | 43.39 |
| 9 | Acid extract†† | 17.03 | 17.74 | 1.73 | 1.88 | 6.80 | 7.37 |
| | Total dry weight‡‡ | 250.41 | 240.82 | 25.36 | 25.48 | | |

*The fractions whose sum gives the dry weight are designated as primary.
†Numbered arbitrarily in the order in which they are described in the text.
‡Halves of the brain.
§Weights of fresh tissue were: H-1, 987.36 mg.; H-2, 945.08 mg.
‖Upper phase from Folch purification.
¶Released by 0.5M NaCl at *p*H 8.8.
**Steps E and F not applied in H-2.
††Extracted by 0.033N HCl in C-M.
‡‡Sum of preceding fractions.

**Table 2.** PERCENTAGES OF PROTEIN RESIDUE (FRACTION 2) IN WET AND DRY TISSUE

| | *Percentage in wet tissue* | | | *Percentage in dry tissue* | | |
|---|---|---|---|---|---|---|
| *Experiment** | *H-1* | *H-2* | *Difference* | *H-1* | *H-2* | *Difference* |
| 29 | 11.09 | 11.54 | 0.45 | 46.36 | 46.27 | 0.09 |
| 30 | 10.97 | 11.03 | 0.06 | 44.01 | 44.76 | 0.75 |
| 31 | 10.87 | 10.87 | 0.00 | 43.94 | 44.48 | 0.54 |
| 32 | 10.92 | 10.92 | 0.00 | 44.03 | 44.43 | 0.40 |
| 33 | 11.13 | 11.09 | 0.04 | 44.57 | 44.19 | 0.38 |
| 35 | 11.13 | 11.10 | 0.03 | 43.90 | 43.54 | 0.36 |
| 36 | 11.22 | 10.88 | 0.34 | 44.34 | 43.52 | 0.82 |
| AVERAGE | 11.05 | 11.06 | 0.13 | 44.45 | 44.44 | 0.48 |

*Experiment 34 was lost in attempts to evaporate at Steps E and G in a Rotary Evapo-mix.

**Table 3.** PERCENTAGES OF MAIN LIPID FRACTION (8) IN WET AND DRY TISSUE

| | *Percentage in wet tissue* | | | *Percentage in dry tissue* | | |
|---|---|---|---|---|---|---|
| *Experiment* | *H-1* | *H-2* | *Difference* | *H-1* | *H-2* | *Difference* |
| 29 | 9.20 | 9.78 | 0.58 | 38.48 | 39.22 | 0.74 |
| 30 | 9.99 | 10.05 | 0.06 | 40.06 | 40.76 | 0.70 |
| 31 | 10.16 | 9.68 | 0.48 | 41.05 | 39.63 | 1.42 |
| 32 | 10.16 | 9.97 | 0.19 | 40.98 | 40.57 | 0.41 |
| 33 | 10.31 | 10.48 | 0.17 | 41.30 | 41.75 | 0.45 |
| 35 | 10.67 | 10.69 | 0.02 | 42.07 | 41.96 | 0.11 |
| 36 | 10.29 | 10.15 | 0.14 | 40.63 | 40.62 | 0.01 |
| AVERAGE | 10.11 | 10.11 | 0.23 | 40.65 | 40.64 | 0.55 |

### Protein Residue, Main Lipid, and Aqueous Fractions

The data (Tables 2–4) on these fractions (2, 8, and 3, respectively) give further confidence in the method.

#### PLP

In the development of the procedure, much effort was devoted to the isolation of PLP. It was reasoned that if myelin is metabolically stable, as Davison believes (*15*), its proteins as well as lipids should have a low rate of turnover, and PLP, labeled during early development, should retain the label into adult life.* It was of primary importance, therefore, that the procedure should provide for the isolation of as much PLP as possible. In the first 20 experiments a modification of the original Folch purification procedure (*29*) was used and PLP was liberated by drying the lower phase under various conditions. The yields of PLP were small. Drying with acetone, as described at Step

*While this work was in progress, Davison (*28*) published data in accord with this prediction.

**Table 4.** PERCENTAGES OF WATER-SOLUBLE FRACTION (3) IN WET AND DRY TISSUE

| | *Percentage in wet tissue* | | | *Percentage in dry tissue* | | |
|---|---|---|---|---|---|---|
| *Experiment* | *H-1* | *H-2* | *Difference* | *H-1* | *H-2* | *Difference* |
| 29 | 1.52 | 1.57 | 0.05 | 6.35 | 6.28 | 0.07 |
| 30 | 1.47 | 1.50 | 0.03 | 5.91 | 6.10 | 0.19 |
| 31 | 1.50 | 1.50 | 0.00 | 6.07 | 6.14 | 0.07 |
| 32 | 1.49 | 1.46 | 0.03 | 6.01 | 5.93 | 0.08 |
| 33 | 1.43 | 1.42 | 0.01 | 5.75 | 5.66 | 0.09 |
| 35 | 1.44 | 1.42 | 0.01 | 5.69 | 5.59 | 0.10 |
| 36 | 1.48 | 1.47 | 0.01 | 5.86 | 5.88 | 0.02 |
| AVERAGE | 1.48 | 1.48 | 0.02 | 5.95 | 5.94 | 0.09 |

F, appeared to give the best results. In the remaining experiments, the technic of Webster and Folch (*22*) was applied to the extract from one of the half-brains (H-1); the lower phase from Step D (Fig. 5) was treated with a solution of citrate, or later, with a solution of NaCl as described at Steps E and F. With this procedure the total PLP is the sum of that released by the NaCl treatment (Fraction 4) and by drying (Fractions 6 and 7). For comparison, Steps E and F were not applied to the extract from the other half-brain (H-2); Step G was applied directly to the lower phase obtained from Step D, and the total PLP is that released by drying. The effect of NaCl treatment was thus tested in seven experiments. In three of these, carried out before the technic had been mastered, the data are suspect for various reasons. In the remaining four, the percentage of total PLP in dry tissue obtained with the NaCl treatment plus drying (H-1) exceeded that found with drying alone (H-2) by an average of 13.3% (range, 11.0-17.1). Of the total PLP obtained with procedure H-1, an average of 62.1% (range, 57.6–66.9) was released by the NaCl treatment.

### Precision of Weighing

The procedure used at Step G affords an opportunity to evaluate the precision of weighing. The main lipid plus PLP fraction (Fraction 5 in the procedure as described, or the lower phase from Step D with procedure H-2) is divided into Fractions 6, 7, and 8, whose sum should equal the weight of the original fraction. In 14 such sets of weighings (two in each of the experiments listed in Tables 2–4), the average recovery was 100.06%, and the maximal deviation was 0.68%.

### Completeness of Lipid Extraction

In many of the experiments, the protein residue (Fraction 2) was digested on a steam bath for several hours in a solution of approxi-

mately 3.5N KOH in 50% ethanol. After acidification and dilution with 50% ethanol, the solution was equilibrated with petroleum ether, the mixture was allowed to stand over night to clarify the phases, the upper phase was removed, and the lower phase was washed 3 times with petroleum ether. The combined petroleum ether solution was washed with 10% ethanol and transferred to a 50-ml. Erlenmeyer flask. The solvent was removed with nitrogen, and the residue was dissolved in a little petroleum ether and transferred through a filter (Fig. 2D (*19*)) into a tared 5-ml. volumetric flask, where it was dried and weighed. A residue equivalent to about 0.4% of the dry tissue was always found. It has not yet been investigated, but it behaves like fatty acid and certainly must represent lipid which is so firmly bound in the brain as to resist the exhaustive extraction procedures which were applied.

### "Strandin" Fraction 9

In four of the experiments listed in Tables 2–4, the two Fractions 3 were pooled for Step H; in the others, each fraction was dialyzed separately. The average of the 10 measurements was 1.25% of Fraction 9 in dry tissue (range, 1.16–1.31). Analyses for sialic acid, hexosamine, hexose, and sphingosine indicated that the fraction is largely strandin, as suggested by its appearance (see above).

### "Acid" Fraction 11

Much effort was devoted to this fraction with the primary objective of providing in the procedure for the isolation of the phosphatido-peptides shown by LeBaron and Folch (*23*) to be extracted by acidified C-M, or of the triphosphoinositides, found by Dittmer and Dawson (*30*) to be the major lipid component of these peptides. Many procedures based on the Folch purification technics were tried without success. A reason for this failure became evident with the publication by Dittmer and Dawson (*31*) of their detailed results. They isolated crude triphosphoinositide equivalent to 3.17–9 mg. of P from 200-gm. portions of ox brain. If rat brain contains corresponding concentrations, less than 0.5 mg. of triphosphoinositide would have been present in the amounts of tissue analyzed in this work, *i.e.*, less than 3% of the weight of Fraction 11.

The early attempts to fractionate the acid extract failed also from another point of view; amorphous, intractable precipitates and emulsions were obtained. These difficulties were overcome in the procedure described in Steps I–M. It consistently yields the following four well-defined fractions.

#### Chloroform-soluble "Acid" Fraction 15

This fraction appears to contain most of the pigment of the tissue. In eleven determinations on half-brains its percentage in dry tissue averaged 0.77 ± 0.09 (S.D.). In five determinations* (four on pooled samples) its fatty acid content was 21.0 ± 2.4%, calculated as stearic acid.

#### Insoluble "Acid" Fractions 18, 19, and 20

These fractions almost certainly are composed mainly of protein. In two experiments the fractions were hydrolyzed in boiling 6N HCl, combined, dried in a rotary evaporator, and redried several times after addition of water to remove HCl. The residues, which were soluble in water, contained 10.4 and 11.2% nitrogen. In 9 half-brains the sums of the three fractions averaged 2.46 ± 0.33 (S.D.) % of dry tissue.

#### Nondialyzable Water-soluble "Acid" Fraction 21

In 9 half-brains, of which two were pooled for Step M, this fraction averaged 0.85 ± 0.15 (S.D.) % of dry tissue. (If one exceptionally low value—0.50%—is omitted, the average is 0.90 ± 0.06%.) This fraction dissolves instantly in water and is exceedingly surface active, so much so that it is difficult to handle in solution because of excessive foaming. Analyses of some of these fractions for a number of substances gave results so variable as to be meaningless. The reason is not known.

#### Dialyzable Water-soluble "Acid" Fraction 22

In 9 half-brains, of which two were pooled for Step M, this fraction averaged 3.40 ± 0.19 (S.D.) % of dry tissue. Analyses of some of these fractions gave the following average percentages (± S.D.): nitrogen, 5.0 ± 0.6; phosphorus, 4.9 ± 0.9; amino acids, expressed as serine, 13.2 ± 1.1; hexose, 2.0 ± 0.9; hexosamine, 0; fatty acids, 0; sialic acid, 0.5 ± 0.1.

### Experiments with Acetate-$C^{14}$

In accord with the purpose of this investigation (see above), at the start and a year later, rats within the first 16 days of postnatal life, were injected intraperitoneally with 31 or 33 $\mu$c of acetate-1-$C^{14}$ or acetate-1,2-$C^{14}$. Six of these rats were used in the experiments carried out in the development of the procedure; in these, the fractions iso-

*An unpublished method of Dr. Herbert L. Meltzer for determination of ester groups was used in these analyses.

lated from the brain were examined for radioactivity. The counting was done in an old, inefficient, end-window counter with the purpose of ascertaining qualitatively whether or not detectable $C^{14}$ was present, but sufficient counts were recorded, usually for five 10-min. periods interspersed with an equal number of background counting periods, to permit statistical analysis of the results. The data will not be presented in detail here; only the principal findings will be summarized. The rats had received labeled acetate 5–15 months before they were used in these experiments. In all of them, most of the fractions examined contained significant radioactivity. The main lipid fraction, Fraction 8, was by far the most active, as expected, but the main protein fraction, Fraction 2, always showed a significant number of counts above background. It is not certain, however, that this radioactivity was in the protein and nucleic acids since the small fatty acid fraction isolated from Fraction 2 always was significantly radioactive.

The "strandin" fraction, Fraction 9, was consistently the most radioactive of all except the main lipid fraction, Fraction 8. This result was unexpected since the gangliosides are not components of myelin where, according to Davison's hypothesis, the metabolically stable lipids of brain are. But the possibility has not been ruled out that the $C^{14}$ of Fraction 9 was present in contaminating lipids from myelin.

Also surprising was the presence of little or no radioactivity in PLP, counted after hydrolysis of the combined fractions with 6N HCl and treatment of the hydroslysate as described for the insoluble "acid" fractions. The amount of material was small, but significant radioactivity was found consistently under the same conditions in considerably smaller fractions, particularly the chloroform-soluble "acid" fraction, Fraction 15, and the fatty acid fraction from the protein residue. The apparent discrepancy between this finding and that of Davison (*28*) may have resulted from a failure of PLP to be labeled by acetate-$C^{14}$ (Davison used glycine-$C^{14}$), but this explanation is difficult to understand. The insoluble "acid" protein fractions, Fractions 18–20, consistently contained small but significant radioactivity. The only other fraction which was consistently free of significant radioactivity was the dialysate, Fraction 10.

The foregoing results are preliminary and subject to revision when a better counting procedure is applied, but they do substantiate the finding by Davison and his colleagues of a remarkable metabolic stability of some substances in the brain and they suggest that the num-

ber of such relatively inert compounds may be greater than has been thought. Perhaps such findings should have been anticipated from the work of Thompson and Ballou (*32, 33*), who found significant concentrations of tritium in all rat tissues studied, including brain, up to a year after exposure of the animals to $H_2{}^3O$.

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# Changes in the Serum Protein Levels During Wound Tissue Regeneration

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**The effect of wounding and formation of regenerating wound tissue on the level of the serum proteins was studied in rats on a protein-free diet. It was found that the level of the globulin component having the third greatest electrophoretic mobility, probably part of the $\alpha_2$-globulin fraction, rose about 60% within 3 days after wounding and did not begin to return to normal until about 10 days later. Concomitantly, the level of the globulin with least mobility, presumably the $\gamma$-globulin component, decreased about 35% and also began to return to normal within 2 weeks after wounding. Varying the rate of healing, by the addition of protein or methionine to the diet, had no effect on the changes in the serum globulin levels. The newly formed $\alpha_2$-globulins were shown to be distinct from fetuin.**

THE RELATIVE AMOUNTS of the protein components in serum have been reported to change considerably during wound tissue regeneration. The most characteristic of these changes appears to be a decreased concentration of albumin and an increase in the $\alpha$-globulins (*1–11*). The relation of these new levels in the serum proteins to wounding or tissue regeneration is obscure. Hormonal factors have been suggested to be involved in altering the level of serum proteins after injury (*4, 22*). Some evidence in support of the idea that the $\alpha_2$-globulins act as detoxifying agents for the metabolic products of the wound debris has been presented (*24*).

Regeneration of wound tissue is accompanied by profound changes in the metabolism of proteins and amino acids. On a protein-deficient diet the rate of tissue regeneration is slower than when an adequate diet is fed (*12–15*). Conversely, the rate of wound tissue regeneration

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can be increased in protein-depleted animals by feeding them a high-protein diet (*16, 17*). If, instead of protein, the sulfur amino acids, methionine or cystine, are supplemented in the protein-deficient diet, the rate of tissue regeneration is similarly increased (*17–20*). It has been shown that proteins containing relatively large amounts of sulfur amino acids accumulate in the regenerating wound tissue (*12*); the amount of these amino acids in the wound tissue increases as the regeneration proceeds (*21*). Thus, the increased amounts of dietary sulfur amino acids appear to be required for adequate wound tissue regeneration to take place; the availability of these amino acids appears to be a limiting factor in determining the rate of healing.

Since the level of the dietary sulfur amino acids is of such basic importance in regulating the rate of regeneration of wound tissue, the question arose as to whether the changes in the serum globulin levels, observed after wounding, are affected also by the availability of these amino acids. If the serum proteins are related in some way to the process of regeneration, changing the rate of healing should be reflected in some change in serum protein level. The present report considers the effects of dietary factors on serum protein composition in wounded animals in which the rate of tissue regeneration has been decreased by protein depletion.

## Experimental

The following experiments were carried out on female albino rats weighing 220 ± 20 gm. at the start of the experiment. The animals were kept on a protein-free diet (*12*) from 3 days prior to wounding until the termination of the experiments. The animals were individually housed after wounding to prevent disturbance of the regenerating tissue. Each animal was offered 8 gm. of the diet daily; this amount of diet was found to be completely consumed in the 24 hr. before the next feeding time. Water was permitted ad libitum. The animals were wounded while under Nembutal anesthesia. A standard wound was made by excising a circular piece of skin and subcutaneous tissue, 4 cm. in diameter, on the back of the neck down to the loose fascia (*10*). Control animals were subjected to the identical treatment except for the wounding.

Blood samples were obtained daily from the tip of the tail. After application of a small amount of xylene, the tail was wiped dry and the skin at the tip end cut off. Approximately 0.1 ml. blood was collected by capillarity in one end of a melting-point tube which had a volume of

about 0.2 ml. The opposite end of the tube was sealed off by heat. The blood was permitted to clot for 15 min. at room temperature and then centrifuged to separate the serum. Samples were discarded if any perceptible hemolysis was observed. On successive days, blood was obtained by removing the scab from the small wound on the tip of the tail. Total protein concentration in the serum samples was determined by the method of Lowry *et al.* (*25*); a pooled sample of lyophilized and reconstituted rat serum was used as the standard. It was noted that the protein concentration in the serum obtained from the tail was 25–30% higher than in serum collected by heart puncture in the same rat at the same time.

Electrophoretic separation of the serum protein fractions was carried out by the horizontal technic in veronal buffer at *p*H 8.6, ionic strength 0.05 (*26*). Cellulose acetate strips were used as the supporting medium (*27, 28*). The serum was diluted with three parts of veronal buffer, resulting in a solution containing approximately 1.8–2.5 gm./100 ml. of protein. About 40 $\mu$g. of protein in a volume of 2 $\mu$l. of solution were applied in a thin streak to the cellulose acetate strip. The electrophoresis was carried out at 4°, using a potential gradient of 20 v/cm. of cellulose acetate strip for 135 min. It was found that by this procedure the proteins in serum samples could be separated into 7 well-defined fractions.

Directly after the electrophoretic separation, the serum proteins on the electrophoretograms were stained by immersion in a solution of 0.2% Azocarmine B in 5% acetic acid for 30 min. (*29*). Excess stain was removed by rinsing the strips in 5% acetic acid for another 30 min.

No attempt has been made to determine the relationship of the amount of dye taken up by each fraction to the actual amount of protein present in each fraction. The purpose of these experiments was not to compare the different protein components in the same serum, but rather to compare corresponding fractions of sera from wounded animals and from the unwounded controls. Therefore, it was not considered necessary to determine the absolute amount of dye which combined with the protein in each fraction.

The intensity of color of the protein fractions on the strips was measured at 580 m$\mu$ by means of a recording densitometer described by McDonald (*30*). The wave length of the incident light of the densitometer was adjusted by means of a grating monochromator; the transmitted light was amplified and traced on a strip chart recorder

through a logarithmic converter. From the areas under the curves of the tracings obtained, the protein concentration was determined. The amount of protein in each fraction was calculated from the equation:

$$P_x = (A_a)\ (P_t)/A_t$$

where $A_a$ is the area under the curve for fraction $a$; $A_t$, the sum of the areas under the curves for all the fractions; $P_t$, grams of protein per 100 ml. of serum as determined by analysis; and $P_x$, grams of protein in fraction.

## Results and Discussion

The electrophoretic pattern of a representative serum sample from a normal animal, as obtained from the strip chart recorder, is shown in Fig. 1. In contradistinction to the five components usually obtained from electrophoresis of serum protein on filter paper, the technique used here separates the serum proteins into seven components. The two extra components probably arise from a further separation of the $\alpha$- and $\beta$-globulins.

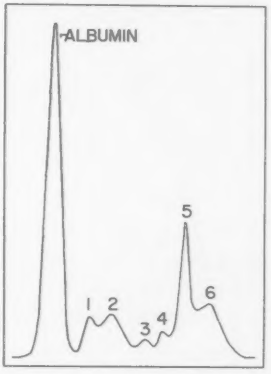


**Fig. 1.** Strip chart recorder pattern of electrophoretically separated fractions of serum proteins on cellulose acetate strips stained with Azocarmine B and color intensity measured in a densitometer at 580 mμ.

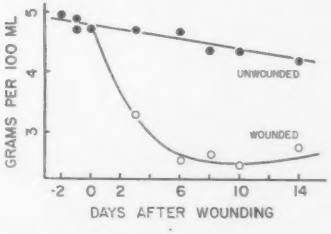


**Fig. 2.** Concentration of albumin in sera (gm./100 ml.) of normal and wounded rats, plotted against days after wounding.

In Fig. 2 are shown the changes in the albumin concentration in the serum from wounded and unwounded rats. It should be noted that the level of albumin in normal rats decreases with time; this is due to the fact that the animals are being maintained on a protein-free diet. The levels of serum albumin in the wounded rats decrease rapidly and significantly beyond the decrease observed in the normal animals. These data are in agreement with previously recorded results (*2–7*).

The third component of the globulin fractions, shown in Fig. 1, increases significantly directly after wounding and only begins to return toward normal after about 10 days. These changes are plotted in Fig. 3. It seems highly probable that this fraction is part of the $\alpha_2$-globulins which have been reported to increase after injury (*2–9*).

Simultaneously with the increase in the globulins in Fraction 3, the level of the globulin component with the lowest electrophoretic mobility decreases significantly and does not begin to return to normal until about 10 days after wounding. The effect of wounding and regeneration on the level of globulin Fraction 6 is shown in Fig. 4. On the basis of mobility, this fraction appears to correspond to the $\gamma$-globulins. Other reports concerning $\gamma$-globulin levels following injury are rather

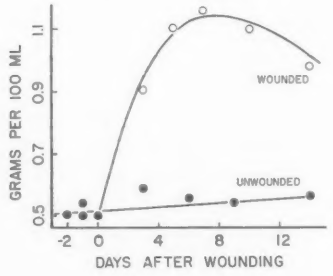


**Fig. 3.** Level of globulin Fraction 3 (gm./100 ml.) in sera of normal and wounded rats, plotted against days after wounding. This globulin fraction is most likely part of the $\alpha_2$-globulins.

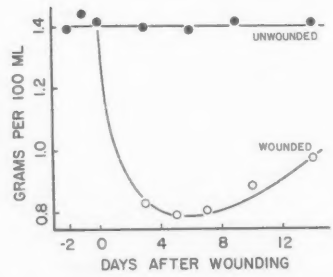


**Fig. 4.** Level of globulin Fraction 6 (gm./100 ml.) in sera of normal and wounded rats, plotted against days after wounding. Mobility of this globulin fraction corresponds to $\gamma$-globulins.

contradictory and inconclusive (*5, 6, 8, 9*). Probst *et al.* (*9*) and Schumacher (*24*) found no change, or only a slight increase in $\gamma$-globulin concentration in the serum following surgical wounds. A questionably significant decrease in $\gamma$-globulin levels, attributed to shifts in body fluids, has been reported after burn injury in humans (*31, 32*) and in rats (*3*).

Since the animals in the experiment reported here had been kept on a protein-free diet, it would be expected that the rate of tissue regeneration in these animals would be slower than if they had been adequately fed (*12–15*). To determine whether the changes in the levels of the serum proteins in wounded rats are influenced by these factors, a comparison was made of the serum protein fractions obtained from wounded rats kept on a protein-free diet and those fed a diet containing 20% casein. It can be seen in Fig. 5 that neither the level of protein in the diet nor the resultant difference in the rate of healing had any effect on the changes in the level of globulin in Fraction 3 and Fraction 6 on the tenth day after wounding; neither were significant differences observed on the sixth and eighth days after wounding. The changes in the albumin level may be attributed to the difference in the protein intake.

Since the effect of the sulfur amino acids on the rate of healing is masked by a high-protein diet (*14*), it was undertaken to study the effect of the sulfur amino acid methionine on the changes in the levels of globulin fractions when a protein-free diet was fed. Control animals received the protein-free diet supplemented with an equal amount of nitrogen in the form of glutamic acid. As shown in Fig. 6 and 7, there appears to be no significant difference in the levels of these globulin fractions after wounding. From these results it is evident that neither the increase in Fraction 3 nor the decrease in Fraction 6, which

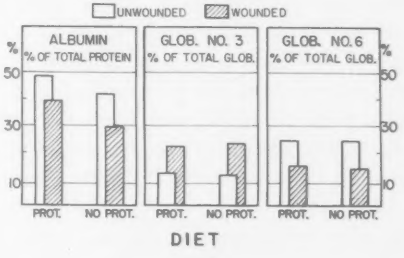


**Fig. 5.** Effect of level of protein in diet on relative concentration of serum proteins on tenth day after wounding.

accompanies injury, is affected by dietary conditions which are able to influence the rate of tissue regeneration. It follows, then, that the changes in serum protein composition which accompany tissue injury are unrelated to processes which control the rate of healing.

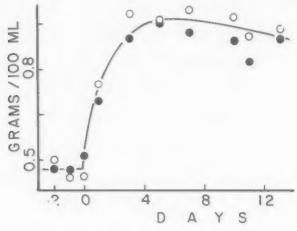


**Fig. 6.** Concentration of globulin Fraction 3 in sera of wounded rats fed a protein-free diet. Open circles indicate 0.002 moles methionine supplemented per 100 gm. diet; solid circles, 0.002 moles glutamic acid supplemented per 100 gm. diet.

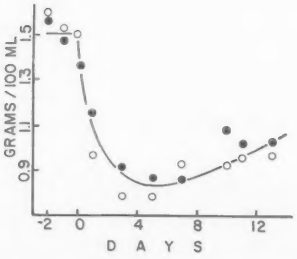


**Fig. 7.** Concentration of globulin Fraction 6 in sera of wounded rats fed a protein-free diet. Open circles indicate 0.002 moles methionine supplemented per 100 gm. diet; solid circles, 0.002 moles glutamic acid supplemented per 100 gm. diet.

It has been reported that fetal serum contains an $\alpha$-globulin component (fetuin) which appears to have growth-promoting properties as tested on the proliferation of cells in tissue culture (*33*). Since the healing of wounds is a growth phenomenon, the possibility that the Fraction 3 globulin might be related to fetuin was investigated. A comparison was made between the electrophoretic patterns of serum from adult wounded and normal fetal rats. It was found that the serum from fetal rats contained a relatively high concentration of a protein with an electrophoretic mobility similar to $\alpha$-globulins. However, this fraction does not correspond in mobility to the Fraction 3 in the serum from wounded rats when fractionated on the cellulose acetate strip. A further difference between the fetuin and the $\alpha_2$-globulins was observed: it was found that the Fraction 3 globulin is precipitable with 0.5 saturated ammonium sulfate, while the fetuin from the serum of fetal rats remained in the supernatant fluid with the albumin.

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# Serum Glycoprotein Levels During Wound Tissue Regeneration

*Martin B. Williamson, Lilita DiLallo, and Harold B. Haley*

**The level of protein-bound hexoses was compared in several fractions of electrophoretically separated globulins in the sera of normal and wounded rats. Changes in the concentration of protein components which were not apparent from the amount of total protein of different fractions could be identified. Although wounding resulted in no change from the normal protein level of one $\alpha$-globulin fraction, the protein-bound hexoses decreased significantly below the normal. In another $\alpha$-globulin fraction from wounded animals, a parallel increase in both protein and hexose above the normal was observed. The total proteins in the $\beta$-globulin fraction were not affected by wounding, even though there was an appreciable increase in hexose in the wounded over the normal level. In spite of the marked decrease in the amount of protein in the $\gamma$-globulins of wounded rats, no significant difference in the hexoses associated with this fraction was observed. Other globulin fractions were unchanged during wound tissue regeneration.**

THE CHARACTERISTIC CHANGES observed in the level of serum protein components after wounding appear to be of relatively minor magnitude when the extensive changes in protein metabolism which accompany the processes associated with wound tissue regeneration are considered (*1–10*). Besides the decrease in the serum albumin, the changes appear to occur almost exclusively in only 2 fractions of the globulins (*9–13*). Since the electrophoretic fractionation of the serum globulins usually yields heterogeneous mixtures of proteins, changes which are not readily apparent might be observable if the various components of each fraction had a characteristic and readily recognizable label. The carbohydrate associated with some of the serum globulins might be used as such an identifying label.

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Trauma has been shown to result in an increase in the total glycoprotein level of the plasma proteins (*14–16*). In particular, the amount of carbohydrate associated with the $\alpha_2$- and $\beta$-globulin fractions of the serum has been reported to be increased after wounding. Neuhaus *et al.* (*17*) have shown that some part of this increase in glycoprotein carbohydrate is due to hexosamine; in the case of the $\alpha_2$-globulins, the increase is reported to be proportional to the increase in the protein of this fraction, while in the $\beta$-globulins the relative proportion of hexosamine increases appreciably. It is apparent, then, that not only the amounts of protein may change in some serum protein fractions, but also, that the relative amount of some of the constituents in each fraction may be altered.

This report presents results which indicate that there are variations in the concentration of protein components found even in those fractions of the serum globulins which ostensibly are not affected by the metabolic changes attendant upon tissue regeneration.

## Experimental

The blood samples used in the following studies were obtained from the tip of the tail of female rats (220 ± 20 gm. at the start of the experiment). The animals were kept on a protein-free diet through the experimental period as described (*6, 9*). Standard wounds were made on the back of the necks of the experimental animals as in previous experiments (*4, 5, 9*).

The blood was collected in capillary tubing. After standing for 15 min. at room temperature, the serum was centrifuged free of the clot and cells. Before use, the serum was diluted to approximately 30 mg./ml. of protein as determined by the method of Lowry (*18*). Several aliquots of the serum proteins were fractionated simultaneously by electrophoresis on separate, horizontally supported, cellulose acetate strips at 4° using a potential gradient of 20 v/cm. for 130 min. After electrophoretic separation, the protein content of the fractions from one aliquot of diluted serum was determined by staining with azocarmine B (*9*). Other aliquots were treated with Schiff-periodic acid reagent in order to determine the hexose content of the protein fractions (*20, 21*). About 60 $\mu$g. of total serum protein was used in the electrophoretic separations which were destined to be used for the determination of protein in each fraction, while about 120 $\mu$g. of protein was used when the glycoprotein hexose was to be measured.

The intensity of the color in each fraction was measured by scanning the electrophoretograms in a densitometer equipped with a log converter to form a tracing on a strip chart recorder (*22*). The measurement of both the protein and the hexose was carried out at a wave

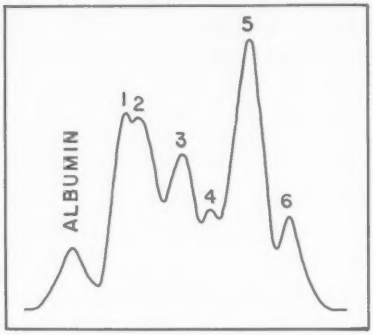


**Fig. 1.** Strip chart-recorder pattern of electrophoretically separated fractions of serum glycoproteins on cellulose acetate strips stained with Schiff-periodic acid reagent. Color intensity measured in densitometer at 580 mμ.

length of 580 mμ. The areas under the resulting curves were taken as a measure of the relative protein or hexose concentration in each fraction. Figure 1 shows the pattern obtained from scanning an electrophoretogram of a representative serum sample (from a wounded rat) which had been treated to measure the glycoprotein hexose.

In order to determine the amount of carbohydrate present in the different fractions of the serum, a calibration curve was prepared. The protein-bound carbohydrate of a composite sample of normal rat serum was determined by the orcinol method (*23*). Different dilutions of this serum sample were applied to cellulose acetate strips. The strips were then subjected to electrophoresis at 20 v/cm. for 5 min. at 4°. After the glycoprotein on the cellulose acetate strip had been treated with the Schiff-periodic acid reagent, the amount of color was measured in a densitometer. These data were then used to construct a calibration curve from which the concentration of carbohydrate of the glycoprotein in the experimental serum samples could be determined. As shown in Fig. 2, a directly proportional relationship exists between the area under the curve of the pattern and the concentration of protein-bound hexose. However, the calibration curve was found not to be reliable when concentrations of less than 0.2 μg. of hexose were involved.

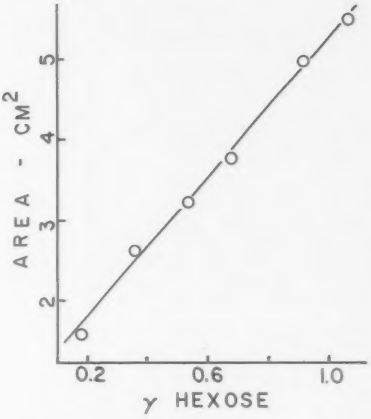


**Fig. 2.** Area under curves obtained from densitometric scanning of electrophoretograms of serum glycoproteins plotted against protein-bound hexose.

## Results and Discussion

The relative distribution of serum proteins among the various fractions on the electrophoretograms was found to be the same as reported previously (*9*). Thus, the serum albumin and the $\gamma$-globulins were found to be significantly reduced during wound tissue regeneration, while the protein in globulin Fraction 3 was increased about 60–80% above the level in the control animals. The other globulin fractions in the wounded animals remained unchanged from those observed in the controls. This is illustrated in Tables 1–4. The total amount of globulins remained constant in both control and wounded animals throughout the experiments, ranging between 5.36 and 6.03 gm./100 ml.

The protein-bound hexose in the albumin fraction (estimated to be about 0.15% of the albumin) was too low to be measured accurately by our method. However, this percentage of carbohydrate is reasonably within the range corresponding to the values reported in the literature (*24*). As might be expected from the fact that the albumin is a relatively homogeneous fraction, no gross change in the proportion of protein-bound hexose was observed after wounding and during the period of tissue regeneration, even though the albumin level decreased appreciably in the sera of wounded rats.

Although no recognizable change occurs in the amount of protein in globulin Fraction 2, a significant decrease in the protein-bound hexose was observed after wounding (Table 1). The difference in the glycoprotein level between the wounded and control rats may be interpreted

**Table 1.** Proteins and Protein-bound Hexoses in Globulin Fraction 2 of Serum from Normal and Wounded Rats

| Days after wounding | % Prot. of total glob. | | % Prot.-bound hex. of total in serum | | Prot.-bound hex. in serum (mg./100 ml.) | | Prot.-bound hex. in glob. 2 (mg./100 mg.) | |
|---|---|---|---|---|---|---|---|---|
| | Control | Wounded | Control | Wounded | Control | Wounded | Control | Wounded |
| 0 | 16.4 ± 1.2 | 15.6 ± 2.5 | 31.5 ± 4.0 | 31.2 ± 2.0 | 25.9 ± 1.6 | 26.4 ± 2.5 | 4.07 | 3.51 |
| 4 | 14.1 ± 0.8 | 14.8 ± 1.6 | 27.1 ± 4.3 | 15.4 ± 3.9 | 22.9 ± 1.4 | 10.9 ± 2.2 | 3.34 | 1.30 |
| 6 | 14.1 ± 1.2 | 15.8 ± 1.6 | 24.2 ± 2.7 | 17.2 ± 2.6 | 26.0 ± 1.8 | 16.6 ± 4.5 | 3.34 | 1.83 |
| 8 | 12.3 ± 1.5 | 15.6 ± 1.4 | 25.1 ± 5.1 | 16.8 ± 2.8 | 27.9 ± 4.8 | 14.9 ± 4.9 | 3.93 | 1.67 |
| 11 | 14.7 ± 0.8 | 14.9 ± 1.5 | 27.3 ± 4.8 | 17.5 ± 2.9 | 21.3 ± 3.4 | 17.0 ± 4.6 | 3.80 | 1.93 |
| 14 | 13.0 ± 1.0 | 13.3 ± 1.1 | 26.4 ± 4.5 | 17.2 ± 3.9 | 28.8 ± 6.0 | 19.3 ± 5.0 | 4.04 | 1.61 |

**Table 2.** Proteins and Protein-bound Hexoses in Globulin Fraction 3 of Serum from Normal and Wounded Rats

| Days after wounding | % Prot. of total glob. | | % Prot.-bound hex. of total in serum | | Prot.-bound hex. in serum (mg./100 ml.) | | Prot.-bound hex. in glob. 3 (mg./100 mg.) | |
|---|---|---|---|---|---|---|---|---|
| | Control | Wounded | Control | Wounded | Control | Wounded | Control | Wounded |
| 0 | 6.7 ± 0.2 | 6.7 ± 1.4 | 8.6 ± 0.9 | 8.6 ± 2.3 | 5.1 ± 1.0 | 4.8 ± 1.8 | 1.42 | 1.20 |
| 4 | 7.4 ± 1.4 | 17.8 ± 2.5 | 14.2 ± 2.1 | 21.1 ± 2.8 | 10.0 ± 4.0 | 17.3 ± 2.1 | 1.95 | 1.50 |
| 6 | 8.7 ± 1.1 | 19.3 ± 4.5 | 16.4 ± 3.7 | 25.8 ± 2.3 | 13.2 ± 3.6 | 31.1 ± 4.5 | 2.32 | 2.95 |
| 8 | 9.4 ± 1.7 | 18.3 ± 1.4 | 17.0 ± 2.6 | 24.0 ± 0.9 | 3.8 ± 1.8 | 26.3 ± 4.3 | 2.55 | 2.63 |
| 11 | 10.0 ± 0.3 | 16.9 ± 1.8 | 13.7 ± 0.6 | 22.4 ± 1.0 | 15.0 ± 5.1 | 26.0 ± 3.5 | 2.46 | 2.92 |
| 14 | 10.6 ± 1.4 | 15.9 ± 2.0 | 16.4 ± 4.1 | 21.8 ± 1.4 | 18.0 ± 2.8 | 27.6 ± 3.7 | 2.78 | 3.13 |

**Table 3.** Proteins and Protein-bound Hexoses in Globulin Fraction 5 of Serum from Normal and Wounded Rats

| Days after wounding | % Prot. of total glob. | | % Prot.-bound hex. of total in serum | | Prot.-bound hex. in serum (mg./100 ml.) | | Prot.-bound hex. in glob. 5 (mg./100 mg.) | |
|---|---|---|---|---|---|---|---|---|
| | Control | Wounded | Control | Wounded | Control | Wounded | Control | Wounded |
| 0 | 31.2 ± 3.4 | 30.9 ± 4.4 | 23.2 ± 3.8 | 23.6 ± 3.4 | 22.7 ± 4.0 | 20.7 ± 4.7 | 1.20 | 1.23 |
| 4 | 30.1 ± 3.5 | 27.1 ± 1.9 | 19.1 ± 2.5 | 21.3 ± 3.3 | 18.0 ± 6.2 | 21.3 ± 5.3 | 1.05 | 1.39 |
| 6 | 28.5 ± 1.0 | 29.9 ± 4.0 | 20.6 ± 1.5 | 24.4 ± 2.4 | 20.2 ± 4.9 | 28.4 ± 3.7 | 1.28 | 1.47 |
| 8 | 29.4 ± 4.9 | 30.6 ± 1.9 | 20.4 ± 1.3 | 29.5 ± 3.0 | 19.6 ± 3.7 | 34.3 ± 3.5 | 1.16 | 1.97 |
| 11 | 27.8 ± 2.5 | 29.8 ± 2.2 | 21.9 ± 3.0 | 28.6 ± 2.7 | 21.2 ± 3.5 | 32.9 ± 2.7 | 1.24 | 2.03 |
| 14 | 27.7 ± 1.9 | 30.9 ± 1.3 | 19.4 ± 2.8 | 26.2 ± 4.1 | 21.6 ± 2.4 | 31.4 ± 4.1 | 1.35 | 2.07 |

**Table 4.** Proteins and Protein-bound Hexoses in Globulin Fraction 6 of Serum from Normal and Wounded Rats

| Days after wounding | % Prot. of total glob. | | % Prot.-bound hex. of total in serum | | Prot.-bound hex. in serum (mg./100 ml.) | | Prot.-bound hex. in glob. 6 (mg./100 mg.) | |
|---|---|---|---|---|---|---|---|---|
| | Control | Wounded | Control | Wounded | Control | Wounded | Control | Wounded |
| 0 | 29.0 ± 5.0 | 28.3 ± 1.5 | 10.0 ± 1.2 | 11.9 ± 0.9 | 6.5 ± 1.7 | 7.1 ± 1.4 | 0.42 | 0.42 |
| 4 | 28.1 ± 3.0 | 20.2 ± 1.3 | 13.2 ± 3.5 | 10.6 ± 1.6 | 7.9 ± 3.2 | 5.4 ± 1.3 | 0.58 | 0.48 |
| 6 | 27.6 ± 3.4 | 14.0 ± 2.0 | 12.0 ± 2.0 | 8.3 ± 1.4 | 8.9 ± 3.1 | 5.5 ± 1.5 | 0.61 | 0.72 |
| 8 | 27.7 ± 2.8 | 15.5 ± 3.5 | 11.2 ± 4.5 | 7.8 ± 0.7 | 7.2 ± 1.5 | 4.4 ± 1.6 | 0.45 | 0.50 |
| 11 | 23.1 ± 2.1 | 15.8 ± 2.6 | 10.4 ± 2.2 | 10.6 ± 2.3 | 4.5 ± 0.6 | 6.4 ± 0.3 | 0.32 | 0.68 |
| 14 | 25.6 ± 2.6 | 18.6 ± 2.8 | 10.1 ± 1.9 | 7.8 ± 2.4 | 8.1 ± 2.2 | 5.6 ± 2.4 | 0.60 | 0.56 |

to mean that (1) a glycoprotein component of this fraction disappears after wounding and is replaced by an equal amount of protein which contains much less, or no, hexose, or that (2) a hexose-containing protein is modified so that it no longer contains the same amount of hexose as in the control animals. These conclusions arise from a consideration of the percentage carbohydrate associated with the proteins in this fraction; in normal animals, the proteins contain an average of about 3.7% hexose, while in the wounded rats the carbohydrate content is considerably below 2%.

As shown in Table 2, the globulin Fraction 3 in the wounded animals rose appreciably above the level in the normal rats, as reported elsewhere (*9, 17*). Not only was this the situation for the protein, but for the protein-bound hexose as well. It should be noted that there also was a significant increase in both the protein and hexose of this $\alpha_2$-globulin fraction in the normal animals, but not to the extent found in wounded rats. In the case of the normal animals, this change can be attributed only to the effect of the protein-free diet. The feeding of a protein-free diet as well as the wounding and tissue regeneration are attended by the appearance of a negative nitrogen balance. This increase in protein and hexose would then seem to be correlated with protein catabolism rather than with the processes which initiate the protein catabolism. Since the negative nitrogen balance after wounding is significantly greater than in normal animals receiving the same protein-free diet (*1, 4*), it might be expected that the amount of protein and hexose in this fraction should be greater in the wounded than in the normal rats (Table 2).

The results in Table 2 may mean either that there is an increase of a component of this serum fraction or that new protein enters the serum which has a higher than normal carbohydrate content. It seems likely that the same glycoprotein is involved in causing the increase in both the normal and wounded animals. This idea is supported by the fact that the new protein appearing in this fraction in the serum of both normal and wounded animals has been calculated to contain about 4.2% hexose.

The data on the proteins in globulin Fraction 5 are presented in Table 3. This was one of the fractions in which the total amount of protein remained unchanged following injury. However, there was a marked increase in concentration of protein-bound carbohydrate in this fraction, to the extent of about 60–80% above the normal. These data may be accounted for in two ways: (1) One protein in this fraction

may be decreased and replaced by an equal amount of another protein with a higher carbohydrate content; the protein with the higher hexose content may be either a new type of protein or merely one which is already present as a component of this fraction. (2) There may be a small increase in the protein level, not detectable by our method of measurement; the additional protein would have to contain a relatively high percentage of hexose.

There is some indication which appears to lend support to the second of these explanations (*17*).

It is particularly important to note that no significant increase in the protein-bound hexose associated with Fraction 5 occurs until about a week after tissue regeneration has begun. This period appears to be crucial in the course of healing in that many biochemical processes appear to change at this time. Thus, it has been shown that: the hexosamine in regenerating wound tissue begins to disappear rapidly at this time (*25*); there is a maximal formation of DNA in the cells of the regenerating wound tissue (*26*); this is the period of highest concentration of RNA and DNA in wound tissue (*26, 29*); a marked change in the type of RNA becomes apparent (*27*); the levels of cystine in the regenerating wound tissue increase markedly (*6*); and appreciable quantities of collagen begin to be deposited in the regenerating wound tissue (*25, 28*).

In globulin Fraction 6, $\gamma$-globulins, (Table 4), the level of protein decreased following wounding. These results are in agreement with our previous experiments (*9*). Within the limits of our method of analysis, there seems to be no significant change in the amount of protein-bound carbohydrate in this fraction. However, since the proteins in this fraction contain so little carbohydrate, the analytical data may not be entirely reliable. Nevertheless, it is apparent that the decrease in the proteins in this fraction must involve molecules which contain very little or no hexose.

No difference in the amount of protein or protein-bound hexose was found in the other globulin fractions of the sera from wounded animals as compared to sera from normal animals.

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# The Direct Spectrophotometric Determination of Protein in Cerebrospinal Fluid

*Roman L. Patrick and Ralph E. Thiers*

**A rapid and sensitive direct spectrophotometric method for the determination of cerebrospinal fluid protein which uses as little as 0.1 ml. of sample has been described. The result is not affected by the ratio of albumin to globulin in the sample.**

**Interfering substances are removed prior to spectrophotometric measurement with an automatic separation chamber employing the principle of gel filtration.**

**Slight alterations of the procedure described for the measurement of protein enable one to obtain the nonprotein moiety for study or to measure proteins in other biologic fluids.**

ACCURATE DETERMINATION of protein in cerebrospinal fluid is hampered by the low concentration of protein present, the relatively small volume of fluid available for analysis, and the presence of relatively large amounts of different types of protein, mainly albumin and globulin. Plum *et al.* (*1*) have reviewed current technics for this determination and commented in detail on their inadequacies. Turbidimetric methods employing reagents such as sulfosalicylic acid give results which depend very markedly upon the relative amounts of albumin and globulin in the sample, since the degree of turbidity obtained with given amounts of these two proteins is quite different. The same difficulty is encountered in most colorimetric methods, along with the added problem that colorimetric reagents measure not only proteins but in many cases nonprotein nitrogenous substances. Unfortunately, the colorimetric procedure which is least affected by differences in the albumin-globulin ratio, that of Lowry (*2*), suffers most seriously from interferences from nonprotein constituents. Zondag and Boetzelaer

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(*3*) have investigated sources of error in this method and state that such a wide variety of nonprotein substances interferes that the procedure should not be used without prior precipitation of protein. However, protein precipitation presents its own problems; it is time consuming and has inherent sources of error including both incompleteness of precipitation and precipitation of interfering substances.

The present study was carried out in an attempt to develop a direct spectrophotometric determination of protein in cerebrospinal fluid which would combine simplicity and speed with accuracy. Protein can be measured spectrophotometrically. Especially at low wavelengths the absorbancy indexes of various proteins are so high as to be more than adequate for direct determination of the small amounts of protein in cerebrospinal fluid. The possible value of this approach has been pointed out by Murphy and Kies (*4*) and Waddell (*5*). Tombs *et al.* (*6*) found that various proteins have similar absorbancy indexes in the range from 200 to 220 m$\mu$. Figure 1 shows the spectra of equal concentrations of serum albumin and gamma globulin. The absorbancy indexes of these two proteins do not differ significantly at 220 m$\mu$. The sensitivity of absorptimetry at 220 m$\mu$ is 10 times that at 280 m$\mu$.

Direct spectrophotometry has been tried for the determination of protein in cerebrospinal fluid. Waddell (*5*) has described such a method, which, however, does not include the removal of interfering substances prior to measurement. Many authors have indicated that cerebrospinal fluid contains various substances such as ascorbic acid, nucleosides, nucleotides, and other unidentified compounds which have absorbancies which would interfere with a direct spectrophotometric

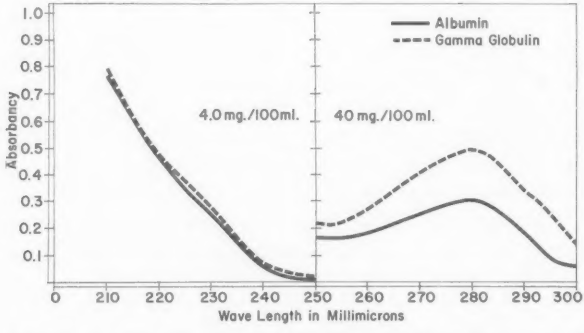


**Fig. 1.** Spectra of equal concentrations of albumin and globulin. Concentration, 4.0 mg./100 ml. from 210 to 250 m$\mu$; 40 mg./100 ml. from 250 to 300 m$\mu$.

determination (*7–9*). Our initial studies verified the impracticality of a direct spectrophotometric method.

The introduction of gel filtration (*10*) has recently made it possible to separate protein from small molecules quantitatively without resorting to precipitation, denaturation, or any other biochemically strenuous technic with the attending hazards. A combination of a rapid technic of separation of proteins by gel filtration and direct spectrophotometry has led to a simple, accurate method for the direct determination of protein in cerebrospinal fluid. This technic does not suffer from interferences nor from incompleteness of measurement of the protein, and it provides results which are independent of the relative amounts of albumin and globulin present in the sample.

## Apparatus

### Separation Chamber

The flow chamber, fabricated from borosilicate glass tubing, is shown in Fig. 2. Its key feature is a constriction to capillary dimensions at exactly the top of as short and thick a cylindrical column of gel as is consistent with a good separation, and with the necessary chamber volume. This capillary performs three functions: it ensures lami-

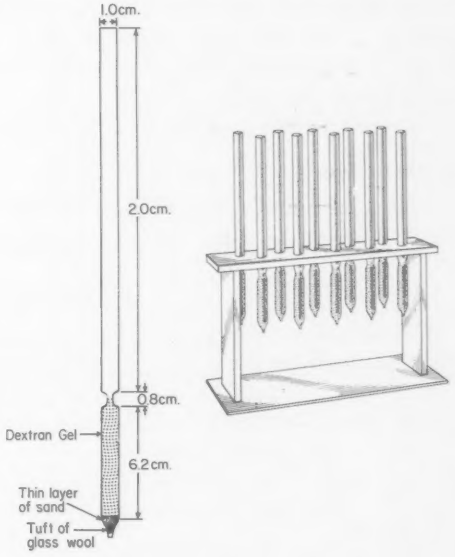


**Fig. 2.** Flow-chamber and stand. Volume of upper section, 20 ml.; volume of lower section, 5 ml.; inside diameter of constriction, 3 mm. The dimension "2.0 cm." should read "20 cm."

nar flow of sample and wash liquid through the chamber, it stops and holds the surface of the diluent so that the chamber does not run dry but holds a reproducible volume of wash liquid at all times, and it prevents dislodgment of gel particles when fluid is added.

The column is filled with dextran as follows. A thin layer of sand is placed on top of the glass wool (Fig. 2) to ensure an even gel bed. The gel, suspended in wash liquid, is then introduced into the chamber, which has been closed at the bottom and filled with wash liquid. The gel particles are allowed to settle by gravity with constant stirring of the unsettled part. The gel bed is finally packed by forcing wash liquid through the chamber under slight pressure. The upper surface of the gel bed is maintained at about the center of the capillary constriction.

The properties of dextran gels (*10*) are such that for a vertical cylindrical flow chamber the relationship of the volume of sample to the chamber volume governs the degree of separation of the protein from smaller molecules, and the volume of wash liquid in which the protein appears (*11*). The dimensions of the device described here are such that it will separate the protein from up to 0.3 ml. of sample and deliver it in up to 3.0 ml. of wash liquid.

### Spectrophotometer

Absorbancy measurements were carried out with a Bausch & Lomb Spectronic 505 Recording Spectrophotometer. Any spectrophotometer capable of making accurate measurements at 220 mμ would be suitable. Fused silica cuvets with a 1.0-cm. light path were employed. These cuvets contained 3.0 ml. of liquid during the measurements.

### Automatic Pipet

A 5-ml. automatic syringe pipet (Scientific Products Co., Catalogue No. P5005) was used for repeated delivery of 2.80-ml. volumes.

## Reagents

The following reagents were used, the first four routinely, as part of the procedure, the last four in original testing of the method.

*Sephadex* Grade G-50, medium particle size was employed, obtained from Pharmacia, Ltd., Upsala, Sweden.

*Lyophilized Serum (Versatol)* This material was obtained from Warner-Chilcott Co., Morris Plains, N. J. It is human plasma treated so as to contain accurately known amounts of protein and other blood constituents.

*Isotonic Saline* 0.85% (w/v) NaCl made from standard reagent-grade material.

*NaF: 2.5% (w/v)* The solution was made from standard reagent-grade materials and was used to prevent fungus growth in the gel.

*Crystalline Bovine Serum Albumin* This was obtained from Armour Pharmaceutical Co., Kankakee, Ill., as a highly purified compound, containing less than .01% of gamma globulin. The nitrogen content of this product has been determined by the Kjeldahl method.

*Gamma globulin* Gamma globulin was obtained from Sigma Laboratories, St. Louis, Mo.

*Tryptophan* Reagent grade tryptophan was obtained from Mann Fine Chemical Co., New York, N. Y.

## Procedure

The protein is separated from compounds of smaller molecular weight in cerebrospinal fluid using the flow chamber shown in Fig. 2 and is delivered directly into the cuvet, dissolved in 3.00 ml. of isotonic saline, by the following steps.

1. Prior to use, fill the upper section of the flow chamber with isotonic saline and allow it to empty itself through the lower section. Discard the washings.
2. Place the spectrophotometer cuvet below the chamber so as to catch the eluate from the gel bed.
3. Place 0.20 ml. of the sample of cerebrospinal fluid in the chamber employing a 1.0-ml. graduated pipet, holding the tip of the pipet against the glass at the constriction of the flow chamber. Allow the sample to pass completely into gel bed before the next step. This takes about 1 min.
4. With the automatic pipet, add 2.80 ml. isotonic saline to the upper chamber and allow it to pass through the gel in the lower chamber. A small plastic tube should be attached to the end of the pipet so that the saline can be placed directly on top of the gel.
5. After this 2.80 ml. of wash liquid has passed completely through the bed, remove the cuvet, mix its contents by covering its top with Parafilm* and inverting 2–3 times. Measure the absorbancy at 220 m$\mu$ using isotonic saline as the reference solution.
6. Convert the absorbancy reading obtained to concentration of

*Marathon Products, Menasha, Wis.

protein in milligrams per 100 ml. using a standard curve determined by measuring absorbancies of dilutions of pure protein which have been treated by the above procedure. Crystalline bovine albumin, commercial protein control serums, or serum of known protein concentration can be employed (Fig. 3).

7. Fill the upper section of the flow chamber with isotonic saline and stopper the outlet by placing a small plastic tip over the lower end. The saline in the upper section of the chamber is left there between uses and is the wash solution used in Step 1 above.

## Evaluation of Procedure

### Precision of Volumetric Recovery from Separation Chamber

The key feature of the separation chamber is its ability to deliver, without attention, exactly that volume of liquid placed in the upper chamber, by retaining a constant volume at all times in the lower chamber. The precision of this feature was tested by pipetting 3.00 ml. of 0.9% saline repeatedly into each of three different flow chambers and collecting and weighing the amount delivered in each case. The results from 12 runs averaged 3.00 ml. and ranged from 2.98 to 3.02 ml., the

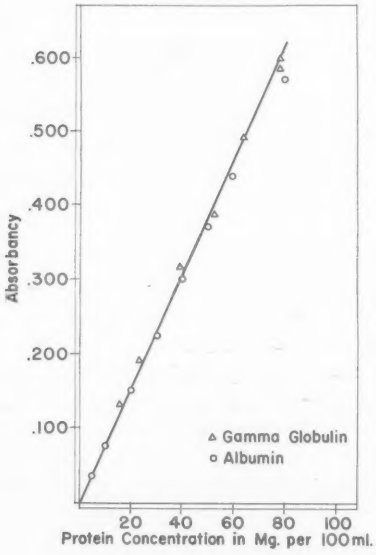


**Fig. 3.** Standardization curve of albumin and globulin: spectrophotometric method.

standard deviation being 0.01 ml. This variation is presumably independent of volume. Upon comparison this proved to be identical to the precision of direct delivery by the same type of pipet.

### Over-all Precision

In order to estimate the precision of the over-all technic, a pool of cerebrospinal fluid was made up, and 11 different determinations were performed on aliquots from this pool using 10 different flow chambers. The data obtained are shown in Table 1. The mean value obtained was 64 mg./100 ml., and the standard deviation of the data was 1.6 mg./100 ml. This experiment was done repeatedly, at different times, and by different analysts. In all cases, the results were not significantly different from those shown in Table 1, and the result of all subsequent work, which has included estimates of precision based upon duplicate data such as those in Table 6, show that the standard deviation of this method is 1.5 mg./100 ml., or about 3% of the result obtained, whichever is greater.

### Accuracy

It is of course impossible to test the accuracy of any method directly on cerebrospinal fluid, since spinal fluids of accurately known protein concentrations are unavailable. Three different technics were chosen to test the accuracy of this method: analysis of commercial control serums, analysis of known amounts of protein to which had been added large amounts of interfering substances, and recovery experiments.

Table 2 shows the results obtained when the lyophilized serum sam-

**Table 1.** ANALYSIS OF REPLICATE SAMPLES*

| *Sample No.* | *Absorbancy (units)* | *Concentration (mg./100 ml.)* |
|---|---|---|
| 1 | 0.504 | 66 |
| 2 | 0.494 | 65 |
| 3 | 0.482 | 63 |
| 4 | 0.472 | 62 |
| 5 | 0.480 | 63 |
| 6 | 0.492 | 65 |
| 7 | 0.482 | 63 |
| 8 | 0.508 | 67 |
| 9 | 0.485 | 64 |
| 10 | 0.485 | 64 |
| 11 | 0.472 | 62 |

*Mean: 64 mg./100 ml.; standard deviation, 1.6 mg./100 ml.

**Table 2.** ACCURACY OF ANALYSIS USING COMMERCIAL LYOPHILIZED PLASMA*

| *Sample No.* | *Concentration taken (mg./100 ml.)* | *Concentration found (mg./100 ml.)* |
|---|---|---|
| 1 | 97 | 96 |
| 2 | 78 | 78 |
| 3 | 58 | 57 |
| 4 | 39 | 39 |
| 5 | 19 | 18 |

*Versatol, Warner-Chilcott Co., Morris Plains, N. J.

ples were analyzed by this method using standard curves made up from Armour Crystalline Serum Albumin. The data indicate that within the precision of the method, the amounts of protein found in the prepared plasma samples correspond exactly with the calculated value.

As described below under *Interferences from Nonprotein Substances,* the results of analysis were identical within the precision of the method, before and after the addition of large amounts of tryptophan to the protein-containing samples.

In recovery experiments known amounts of protein ranging from 100 to 300 $\mu$g. were added to aliquots of pooled cerebrospinal fluid in which the protein concentration had previously been determined. The resulting data are shown in Table 3. The percentage recovery ranged from 91 to 106, with a mean recovery of 99%. These data show no deviations in accuracy beyond those expected from the measured precision of the method.

### Interference from Nonprotein Substances

An experiment was performed to determine whether a nonprotein, the amino acid tryptophan, would interfere with the measurement of

**Table 3.** RECOVERY OF ALBUMIN ADDED TO A SAMPLE OF NORMAL CEREBROSPINAL FLUID*

| *Sample No.* | *Micrograms taken* | *Micrograms recovered* | *Percentage recovered* |
|---|---|---|---|
| 1 | 100 | 102 | 102 |
| 2 | 150 | 137 | 91 |
| 3 | 200 | 213 | 106 |
| 4 | 200 | 204 | 102 |
| 5 | 300 | 295 | 98 |
| 6 | 300 | 284 | 95 |

*Mean recovery 99%.

protein with this procedure. Tryptophan has a very high absorbance at the wavelength employed for measurement of the protein. A sample of cerebrospinal fluid was taken which contained 960 $\mu$g. of protein as determined by this method, and to it 450 $\mu$g. of tryptophan was added.

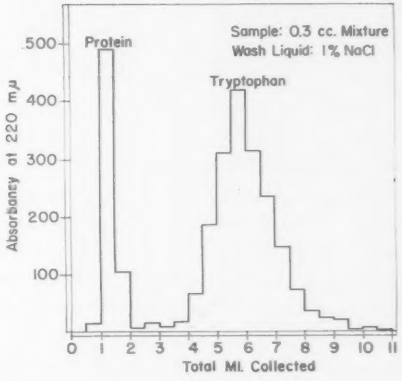


**Fig. 4.** Separation of protein from tryptophan.

Aliquots of this combined solution were treated by the procedure described above except that in some cases the effluent from the column was divided into much smaller portions and the absorbancy of the effluent was measured at frequent intervals throughout the discharge of the protein and during the washing step. Figure 4 shows the results obtained when an aliquot containing 60 $\mu$g. of protein was run. The separation of protein from nonprotein materials was in no way altered by the presence of the tryptophan, and 58 $\mu$g. of protein was recovered. In all such experiments, the recovery of the protein was complete within the precision of the method.

### Comparison of Biuret and Spectrophotometric Methods

For some time, both the method described here and the standard biuret method (*12*) were routinely run on every sample of spinal fluid obtained in the Clinical Chemistry Laboratory. Table 4 shows the results for 25 of these spinal fluids. The results for the spectrophotometric method are consistently lower than those for the biuret method, as would be predicted from the higher specificity of the former technic. In some cases, these differences are so great as to encourage different clinical interpretations.

**Table 4.** Comparison of Cerebrospinal-fluid-protein Data Using Spectrophotometric and Biuret Methods (mg. per 100 ml.)

| *Sample No.* | *Biuret* | *Spectrophotometric* | *Difference* |
|---|---|---|---|
| 1 | 105 | 86 | −19 |
| 2 | 37 | 42 | + 5 |
| 3 | 76 | 80 | + 4 |
| 4 | 35 | 20 | −15 |
| 5 | 105 | 87 | −18 |
| 6 | 75 | 47 | −28 |
| 7 | 45 | 24 | −21 |
| 8 | 44 | 35 | − 9 |
| 9 | 45 | 27 | −18 |
| 10 | 56 | 47 | − 9 |
| 11 | 52 | 32 | −20 |
| 12 | 46 | 29 | −17 |
| 13 | 62 | 61 | − 1 |
| 14 | 48 | 40 | − 8 |
| 15 | 39 | 33 | − 6 |
| 16 | 57 | 33 | −24 |
| 17 | 58 | 36 | −22 |
| 18 | 57 | 42 | −15 |
| 19 | 52 | 45 | − 7 |
| 20 | 48 | 27 | −21 |
| 21 | 50 | 31 | −19 |
| 22 | 43 | 30 | −13 |
| 23 | 78 | 46 | −32 |
| 24 | 54 | 21 | −33 |
| 25 | 84 | 78 | − 6 |

### Direct Spectrophotometry on Cerebrospinal Fluid

Direct spectrophotometry has been recommended by at least one group of workers for the determination of cerebrospinal fluid proteins (*6*). The feasibility of direct spectrophotometry was tested by an experiment wherein the absorbancies of two identical aliquots of spinal fluid were measured. One of these two samples was simply diluted to exactly 3.00 ml. The other was passed through the flow chamber by the procedure described above, and the protein fraction only was diluted by this procedure to 3.00 ml. The results are shown in Table 5. Clearly, data obtained by direct spectrophotometry are considerably too high and are not suitable for use in determining cerebrospinal-fluid protein. Correction technics involving measurements at more than one wavelength (*5*) were also tested and found to be quite ineffective in reducing the error due to nonprotein-absorbing substances.

**Table 5.** Effect of Interfering Substances on Estimation of Protein by Direct Spectrophotometry

| | *Absorbancy at 220 mμ* | |
|---|---|---|
| *Sample No.* | *Aliquot diluted to 3.00 ml.* | *Protein fraction of aliquot, in 3.00 ml.* |
| 1 | 0.603 | 0.455 |
| 2 | 0.393 | 0.250 |
| 3 | 0.462 | 0.284 |

## Normal Range

The determination of the normal range for constituents in cerebrospinal fluid is rendered difficult by the inherent problems in collecting samples of spinal fluid from healthy individuals. Samples drawn from patients at the time of spinal anesthesia have generally been utilized, but these samples clearly do not come from people who are normal in the same sense as does a blood sample drawn from a healthy individual. For lack of a more definitive approach, at least at present, proteins were determined by the procedure described on 14 patients prior to surgery. This group of patients was selected at random by several anesthesiologists. These data are shown in Table 6 and, while they do not purport to establish a normal range for this method, they clearly demonstrate that this procedure does not disagree with the findings of others.

**Table 6.** Duplicate Protein Determinations on Cerebrospinal Fluid from Patients Undergoing Spinal Anesthesia (mg. per 100 ml.)

| *No.* | | |
|---|---|---|
| 1 | 32 | 34 |
| 2 | 32 | 34 |
| 3 | 37 | 34 |
| 4 | 42 | — |
| 5 | 43 | 47 |
| 6 | 26 | 27 |
| 7 | 30 | 31 |
| 8 | 47 | 46 |
| 9 | 45 | 44 |
| 10 | 39 | 40 |
| 11 | 37 | 38 |
| 12 | 49 | 49 |
| 13 | 45 | 44 |
| 14 | 59 | 58 |

## Discussion

In routine use this method has decided advantages as compared with other methods. It involves no reagent other than saline. The substance to be determined is measured directly without interference from other substances. The separation procedure requires no attention from the operator after the sample and isotonic saline have been added. The cuvets are collected for spectrophotometry at the convenience of the technician since the separation chambers automatically deliver the protein and only the protein in exactly 3.00 ml. of isotonic saline. The precision of this delivery is identical to that of the delivery from a 5-ml. Mohr pipet (*11*). The measurement of protein at 220 mμ is highly sensitive. As little as 15 μg. in the 3.0 ml. can be measured. The determination is not significantly affected by the albumin-globulin ratio of the cerebrospinal fluid. Albumin and gamma globulin have standard curves which are very close together, and either might be used for standardization of this method. As demonstrated above, the commercial lyophilized serum may also be used for standardization.

The precision of the method does not depend on the precision with which the chambers are constructed or the volume of the gel. Chambers with volumes from 4.7 to 5.2 ml. gave identical results, within the precision of the method.

The sample used need not be 0.20 ml. Any sample volume up to 0.3 ml. is satisfactory. For cases where smaller amounts of sample are available, such as in pediatric work, 0.10 ml. may be used. The volume of wash liquid is merely adjusted so that the final volume of wash liquid plus sample is 3.00 ml., since it has been determined that this volume contains all of the protein when a column of the size described is employed. Even higher sensitivity can, of course, be obtained by slight modifications of the method such as utilization of spectrophotometer cuvets with longer light paths, or measurements at wavelengths lower than 200 mμ.

A slight variation in the method enables one to study the nonprotein constituents of cerebrospinal fluid. Several authors have reported that cerebrospinal fluid has an absorption peak at 265–275 mμ (7–9, 13–15). We have found that the constituent of cerebrospinal fluid which is responsible for this peak remains in the flow chamber along with the small molecules rather than being washed out by the 3.0 ml. which contains the protein. These constituents can be removed from the chamber by washing with at least 12 ml. of normal saline. The results of a

study of the nonprotein fraction of cerebrospinal fluid will be the subject of a subsequent report.

In routine application of this method, we have found it helpful to utilize 10 different flow chambers in a small plastic rack, allowing 10 simultaneous separations. When not in use, the chambers are filled with isotonic saline to prevent drying of gel particles on the upper surface of the gel bed. The lower end is stoppered with a small plastic tube. These chambers have been used repeatedly with the original filling of dextran for periods up to 8 months without showing signs of deterioration. During the summer months, several of the chambers became contaminated with fungi when they were not used for a few days. It was found that a 2.5% solution of NaF prevented the growth of these organisms, and this solution was therefore kept in unused chambers.

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# Factitiously Low Amylase Values

*Sylvan M. Sax and George E. Trimble*

**When a commercial substrate was used in the determination of serum amylase activities of patients with acute pancreatitis, results much higher than those reported by the manufacturer with similar patients were found. A failure to quantitate all of the reducing groups liberated during incubation may account for the lower results. A modified saccharogenic method has been developed, which, by increasing the ratios of starch to enzyme and copper to reducing sugars, considerably extends the range of the determination. Unequivocally elevated serum and urine amylase values can be measured without repetition of all or part of the test and with good precision.**

RECENT STUDIES of saccharogenic amylase methods (*1, 2*) stress the importance of using sufficient substrate to maintain maximum reaction velocity during incubation. This paper presents evidence that commonly used spectrophotometric copper reduction methods for the determination of reducing groups in postincubation mixtures have been misapplied, leading to factitiously low amylase values. A modified saccharogenic method, which permits analysis of most pathologic human sera or urines without repetition of either incubation or copper reduction steps and without sacrifice of precision in the normal range, is described.

## Materials

Methyl and propyl *p*-hydroxybenzoates were obtained from Eastman Organic Chemicals Department, tris (hydroxymethyl) aminomethane ("Sigma 121" or Tris) from Sigma Chemical Co., soluble starch (Lintner) and *D*-glucose from Fisher Scientific Co., and *D*-maltose hydrate from Nutritional Biochemicals Corp. Krautman's Stable

From the Department of Pathology, The Western Pennsylvania Hospital, Pittsburgh 24, Pa.

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Buffered Starch and Krautman's Special Deproteinizing Reagent were purchased from U. S. Scientific Co., Brooklyn, N. Y. All other chemicals are ACS reagent grade.

Blood and urine specimens from hospitalized patients and Abnormal Clinical Chemical Control Serum (Hyland Laboratories) served as sources of high and normal amylase activity.

Solutions used in this study are as follows:

*Glucose or maltose standards.* Dissolve the desired quantity of *D*-glucose or *D*-maltose hydrate in 0.02 *M* benzoic acid-sodium benzoate buffer, *p*H 5.2.

*5% Zinc sulfate* (*3*)

*0.3 N Barium hydroxide* (*3*)

*Somogyi copper reagent* (*3*)

*Arsenomolybdic acid reagent* (*3*)

*10% Sodium tungstate* ($Na_2WO_4 \cdot 2\ H_2O$)

*0.667 N Sulfuric acid*

*Folin-Wu copper reagent* (*4*)

*Phosphomolybdic acid reagent* (*4*)

*Henry-Chiamori starch substrate* (*1*)

*1 M Tris-HCl buffer, pH 7.2* Flocculation was observed after several weeks at room temperature. The solution is stable at 4°. 1 *M* Tris-HCl buffers of other *p*H values were also prepared for running a *p*H-activity curve.

*0.1 M Tris-HCl buffer, pH 7.2*

*0.1 M Phosphate buffer, pH 7.0* (*1*)

*Substrate A.* Heat 950 ml. of 0.1 *M* phosphate buffer, *p*H 7.0 to boiling. Add a slurry of 15 gm. of soluble starch, 1.35 gm. of methyl *p*-hydroxybenzoate, 0.27 gm. of propyl *p*-hydroxybenzoate, and 2 gm. of NaCl in 35 ml. of cold buffer and remove heat. Use an additional 15 ml. of cold buffer to transfer any remaining slurry. Stir, cool, and discard scum. The concentrations of preservatives are those used by Marsters *et al.* (*5*).

*Substrate B.* Prepare the same as *A*, but omit NaCl and substitute 0.1 *M* Tris-HCl buffer, *p*H 7.2 for phosphate buffer.

*Substrate C.* Prepare the same as *A*, but omit NaCl and substitute water for phosphate buffer. Store at room temperature and discard after one month.

## Methods

In addition to modifications described later, the method of Folin and Wu (*4*), its Tonks modification (*6*), and the method of Nelson and

Somogyi as described by Annino (*3*), were used to determine reducing sugars.

In addition to modifications described later, the amylase methods of Henry and Chiamori (*1*), Krautman (*7*), and Somogyi (*8*) were used. With all three, reducing sugars were determined by the Folin and Wu method (*4*). In applying the Henry and Chiamori and Somogyi procedures, incubation was repeated with less enzyme solution if results over 700 units were observed; copper reduction was repeated with less deproteinized incubation mixture if results over 400 mg./100 ml. of glucose equivalent were observed. The Krautman procedure utilizes a starch substrate and deproteinizing reagent whose exact compositions are undisclosed. Whereas incubation and deproteinization are described in stepwise fashion, the directions state only that the deproteinized incubation mixture is subjected to a blood sugar determination "in the usual way." No limiting value beyond which either the copper reduction or incubation steps should be repeated is stated. In applying the Krautman method, unless otherwise indicated, only one incubation and one copper reduction was carried out per specimen.

All incubations were at 40 ± 0.1°. Measurements for *p*H were made at 25° with a Radiometer *p*H Meter 22.* Spectrophotometric measurements were made with a Bausch and Lomb Spectronic 20 spectrophotometer† in ½-inch tubes. When absorbance readings exceeded 0.7, solutions were diluted with reagent blank or water and reread. Water was substituted for reagent blank as a diluent only after establishing that the slope and linearity of the standard curve remained unchanged and that the stability of the colored product was not adversely affected.

## Results

### Determination of High Amylase Activities with a Commercial Kit

This experiment was intended to clarify two puzzling features of the Krautman procedure. First, an incubation time of 45 min., instead of the usual 30 min., is used. In addition, the following note appears: "The serum of 56 known acute pancreatitis patients yielded results from 189 to 300 units of amylase; 150 blood serums from known normal patients yielded results from 70 to 160 units." (*7*) Since the Krautman unit is calculated like the commonly employed Somogyi saccharogenic unit (*8*), one should anticipate results 50% higher by the Kraut-

*The London Company, Cleveland 11, Ohio.
†Bausch & Lomb Incorporated, Rochester, N. Y.

**Table 1.** Comparison of Amylase Activities in Abnormal Patients' Sera as Determined by Krautman and Henry & Chiamori Methods

| Serum | Glucose Conc. mg.-% (Folin-Wu) | Amylase (mg.-% Glucose Equivalent) Method A* | Method B** | Method C*** | C/A |
|---|---|---|---|---|---|
| 1. | 339 | 710 | 29 | 910 | 1.28 |
| 2. | 145 | 1020 | 238 | 1480 | 1.45 |
| 3. | 190 | 760 | 152 | 1150 | 1.51 |
| 4. | 124 | 480 | 234 | 640 | 1.33 |
| 5. | 105 | 1080 | 294 | 1450 | 1.34 |
| 6. | 130 | 630 | 257 | 960 | 1.52 |

* Method A - Henry and Chiamori (1)

** Method B - Krautman (6)

*** Method C - Single Krautman incubation, but reducing sugars assayed on smaller aliquots of filtrates to insure complete quantitation. (see text)

man than by other methods which employ 30-min. incubation. However, other saccharogenic methods produce values in excess of 300 units when applied to pathologic sera (*9*).

Parallel amylase determinations, by the methods of Henry and Chiamori and Krautman, were performed on 6 patients' sera in which high activity had previously been demonstrated amyloclastically. Results in Table 1 show much lower values by Krautman's method (Column B), however, especially in hyperglycemic sera; when the sugar determination was repeated on smaller aliquots of the same deproteinized incubation mixtures, the values shown in Column C were obtained. These exceed the Henry and Chiamori results by an average of somewhat less than 50%. Analysis of two of the same sera by the Somogyi method gave results in good agreement with those in Column A. Repetition of two of the Krautman determinations with different batches of commercially prepared substrate and deproteinizing reagent yielded practically the same values shown in Columns B and C.

Next a Hyland Abnormal Clinical Chemical Control Serum, having a labeled amylase activity of 925 Somogyi units per 100 ml., was incubated with Krautman's Starch in a series of test tubes. Krautman's Deproteinizing Reagent was added at 5-min. intervals from 0 to 60 min. and reducing sugars determined in the filtrates by the Folin and Wu procedure. Copper reduction was repeated on smaller amounts of filtrate when glucose equivalents greater than 400 mg./100 ml. were obtained. The resulting curve (Fig. 1), is linear to more than 800 mg./100 ml. Repetition with a different batch of the commercial substrate gave

virtually a superimposable curve. From these results and for reasons discussed later it seems reasonable to attribute the low amylase activities observed by Krautman to the procedure or procedures used for the determination of reducing sugars.

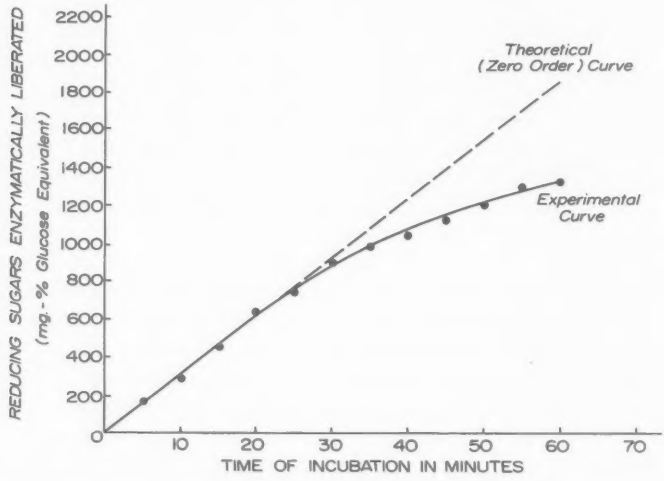


**Fig. 1.** Measurement of rate of liberation of reducing sugars from Krautman's substrate by a high-activity control serum.

## Copper Reduction Methods

The copper reduction method used to analyze incubation mixtures should, without repetition on smaller aliquots, measure the sum of reducing groups liberated to the point at which their rate of formation decreases, glucose originally present in strongly hyperglycemic sera, and reducing sugars originally present in the substrate. Thus if zero-order amylolytic activity extends beyond 1000 units per 100 ml. of enzyme solution, a suitable sugar method should measure more than 1500 mg./100 ml. of glucose equivalent without repetition on smaller aliquots, while retaining precision in the range of low sugar concentrations. For the determination of urine amylase activities, an even wider range sugar method is desirable. The Folin-Wu and Nelson-Somogyi standard curves at high glucose concentrations are shown in Fig. 2. Leveling is observed near 400 mg./100 ml. in the original and modified Folin-Wu curves and below 800 mg./100 ml. in the Nelson-

Somogyi curve. None of these methods therefore covers the desired range.

The useful portions of the curves can be extended by increasing the ratio of copper to reducing sugar, either by using more copper or less

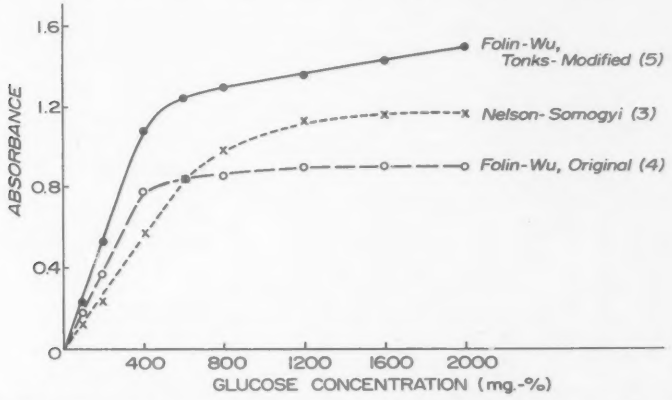


**Fig. 2.** Appearance of glucose standard curves up to 2000 mg./100 ml. as run by three commonly used blood glucose methods.

sugar solution. The loss in sensitivity accompanying the latter alternative might be obviated by either omitting the final dilution (*10*) or by measuring absorbance at a higher wave length (*11*). Both possibilities were examined.

A Tonks curve was run using one-fifth the amount of standards and omitting the last dilution, making a final volume of 6 rather than 25 ml. A similarly modified Nelson-Somogyi curve was run (final volume 4 rather than 25 ml.). Results shown in Fig. 3 indicate good linearity for the latter, slightly less so for the former. The modified Nelson-Somogyi curves were read at 490 (*3*) and at 540 mμ (*10*). The higher wave length provides greater sensitivity without sacrificing linearity and without appreciably elevating the absorbance of the blank. Additional glucose standards of higher concentration were run and no appreciable deviation from the Beer-Lambert law was observed below 4000 mg./100 ml. The added sensitivity afforded by measuring absorbance at 540 mμ permits a further reduction of the amount of sugar solution taken for assay by a factor of 2, thus extending the linear portion of the curve to over 8000 mg./100 ml. In Fig. 3, 4, and 5, the ordinate represents the product of the observed absorbance and the dilution factor.

Next, the Nelson-Somogyi modification suggested by Teller (*11*) was tried. The volumes of glucose standards, Somogyi copper reagent, and arsenomolybdic acid reagent were in the ratio of 0.02:1:1 ml., in a final dilution of 25 ml. Spectrophotometric measurements at 660 mμ

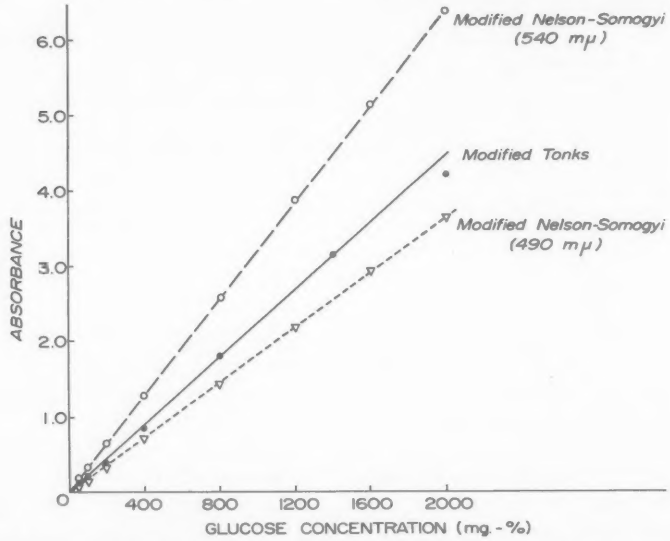


**Fig. 3.** Glucose standard curves obtained from Nelson-Somogyi and Tonks procedures, modified by using one-fifth the quantity of glucose and omitting final dilution.

produced the curve in Fig. 4. The relationship between concentration and absorbance is virtually linear throughout the range. However, the sensitivity (change in absorbance per unit increase in concentration) is rather low. These results indicate that, although any of the Tonks or Nelson-Somogyi modifications described in this section might fulfill the previously noted criteria for suitability in the amylase determination, the procedure in which the final dilution step is omitted and spectrophotometric measurements are made at 540 mμ appeared most promising.

### Development of Extended-Range Amylase Procedure

Next, application of the extended-range Nelson-Somogyi modification to the determination of reducing substances in deproteinized incubation mixtures was attempted. Tentative decisions concerning sub-

strate and buffer concentrations and the nature of the buffer and deproteinizing agents had to be made at this time. Since deviation from zero-order kinetics is anticipated near 700 amylase units in either the Henry and Chiamori or Somogyi incubation mixtures (*1, 2*), and since

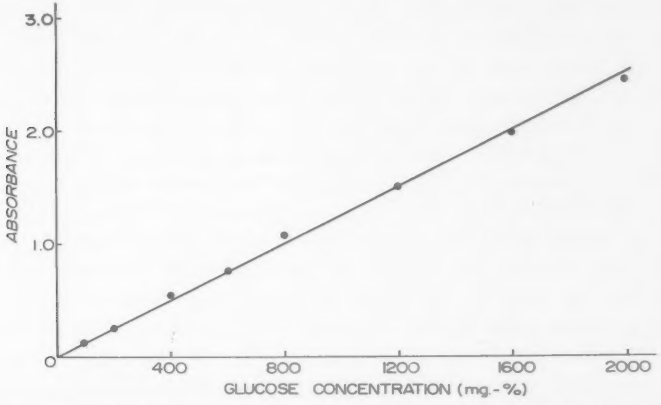


**Fig. 4.** Glucose standard curve obtained from Nelson-Somogyi procedure, modified by using one-fifth the quantity of glucose and making spectrophotometric measurements at 660 mμ.

considerably higher activities are not infrequently observed, the ratio of starch to serum or urine was increased from 75 to 150 mg./ml., thereby approximately doubling the linear reaction range. Since the need for adequate buffering, particularly for urinary amylase determinations, has been demonstrated (*1*), Substrate A was used in early experiments. Alkaline zinc deproteinization was considered the method of choice since clearer filtrates are obtained (*3*), nonsugar-reducing substances in urine are effectively removed (*11*), and the required reagents are available in laboratories performing the Nelson-Somogyi procedure.

As a prelude to attempting actual incubations, a glucose standard curve was run in the presence of substrate and deproteinizing reagents. Centrifugation of mixtures of 0.1 ml. of glucose standards, 1 ml. of Substrate A, 2 ml. each of 5% zinc sulfate and 0.3 *N* barium hydroxide, and 4.9 ml. of water gave clear supernatants. Halving the amounts of deproteinizing reagents resulted in somewhat turbid supernatants. Mixtures of 1 ml. each of supernatants, water, and Somogyi copper reagent were heated in a boiling water bath for 15 min.

Addition of 1 ml. of arsenomolybdic acid reagent produced clear solutions. Spectrophotometric readings were made at 540 mμ. The resulting curve showed a slight upward concavity and about 13% decrease in slope compared with that obtained with aqueous standards. Repetition with Substrate B produced full color and a linear curve, but a very high blank. Using Substrate C and replacing 0.1 ml. of water with an equal volume of 1 *M* Tris-HCl buffer, *p*H 7.2, a curve of identical shape and slope was obtained, but with a much lower blank. Therefore, in subsequent work, buffer and substrate were prepared and stored separately. Another standard curve was run in the presence of 1.8 instead of 1 ml. of Substrate C. Although good linearity and a low blank were obtained, some turbidity was noted in the final colored solutions.

A maltose standard curve, run in the presence of Substrate C, tris buffer, and deproteinizing reagents, is shown in Fig. 5. Unlike Henry and Chiamori's concave curve (*1*), a nearly straight line was produced throughout the range. Anhydrous maltose, as compared to an equal weight of glucose, is capable of reducing 47% of the amount of cupric reagent under the described conditions. In another experiment in which the reaction time for the copper reduction was varied, 15 min. in an actively boiling water bath was found to be adequate for maltose. This result does not rule out the necessity for prolonging the heating cycle to oxidize higher molecular weight sugars (*2*); nevertheless the 15-min. reaction time was adopted and has proved satisfactory when applied to incubation mixtures.

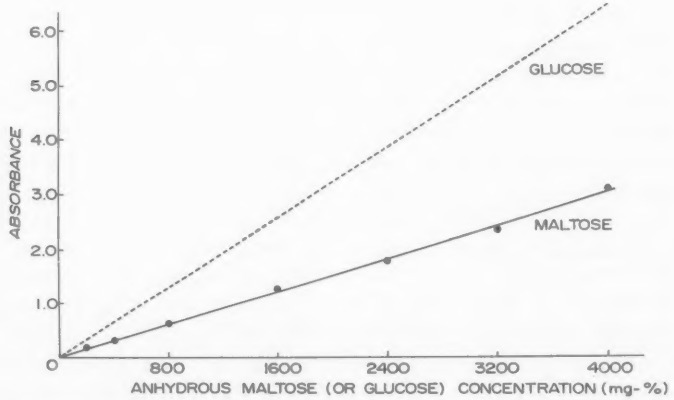


**Fig. 5.** Maltose standard curve run in presence of substrate and deproteinizing reagents.

Tubes containing 1 ml. of day-old Substrate C, 0.1 ml. of 1 *M* Tris-HCl buffer, *p*H 7.2, and 0.1 ml. of commercial control serum having a labeled activity of 925 Somogyi units, were incubated at 40° for times varying from 0 to 90 min. At zero time and at 10-min. intervals, the re-

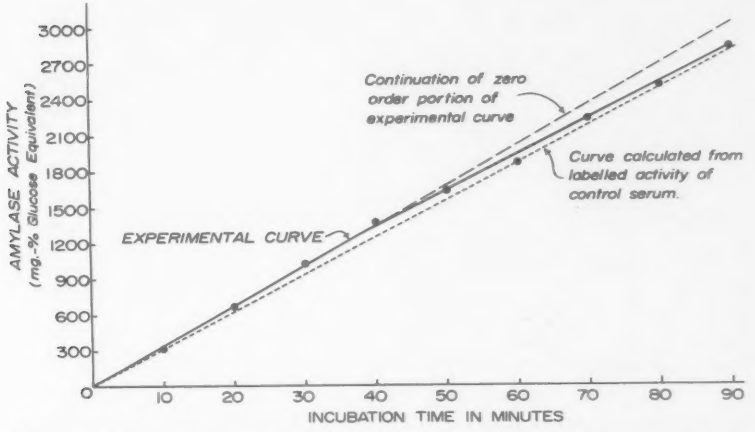


**Fig. 6.** Relationship between enzyme activity of a commercial control serum and incubation time.

action was stopped by zinc deproteinization. Quantitation of reducing sugars by the modified Nelson-Somogyi technic described above produced the plot shown in Fig. 6. The liberation of reducing groups proceeded at a constant rate to about 1400 mg./100 ml. of glucose equivalent, as expected (*1*). Thereafter the rate decreased sufficiently slowly to permit measurement of activities up to 3000 mg./100 ml., with an error of less than 8%. Upon repetition of this experiment with 45-day-old substrate, which showed no evidence of mold or bacterial decomposition, the same initial velocity was observed, although the rate began to drop sooner (at about 1000 mg./100 ml.) and more steeply. The activity loss may possibly be attributed to a decrease in concentration of available substrate, resulting from micelle formation (*13*). It is recommended, therefore, that the substrate not be stored for longer than one month.

The change from phosphate to Tris buffer improved the quantitation of reducing groups. Before using Tris in the amylase assay, additional data had to be obtained, namely the determination of *p*H of

maximum activity, comparison of reaction rates in the two buffer systems, and measurement of buffering capacity. A *p*H-activity curve was run by incubating 0.1 ml. of highly active serum for 30 min. at different *p*H's with 1 ml. of Substrate C and 0.1 ml. of 1 *M* Tris-HCl

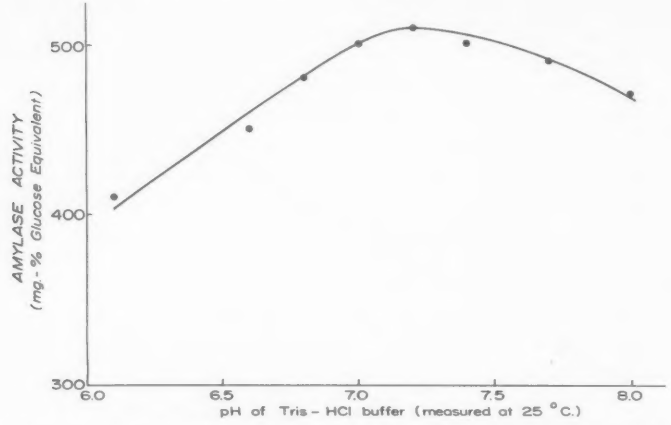


**Fig. 7.** Amylase activity as a function of the *p*H of the Tris-HCl buffer used in the incubation mixture.

buffer. The resulting curve (Fig. 7) is skewed upward and has a rather broad maximum centering about *p*H 7.2. In another experiment several sera and strongly acidic or alkaline urine specimens were mixed with 1 ml. of Substrate C and 0.1 ml. of 1 *M* Tris-HCl buffer, *p*H 7.2; the *p*H values of the incubation mixtures were measured before and, in some cases, after incubating for 30 min. at 40°. The *p*H values of mixtures of equal volumes of supernatants after deproteinization, water, and Somogyi copper reagent were also recorded in several instances. Data summarized in Table 2 indicate that buffering is probably adequate under the most extreme conditions likely to be encountered in routine work, despite the *p*H of incubation being somewhat more remote from the *p*K of the Tris than from the phosphate system. In another experiment, initial velocities were determined with the same serum, with freshly prepared starch substrate, and with a constant chloride concentration, in the presence of either Tris or phosphate buffers of the same molarity. In measuring reducing groups, internal glucose standards were used to compensate for the decreased color developed

**Table 2.** Evaluation of Buffering Capacity of Tris-Hydroxymethyl-aminomethane under Conditions of Proposed Amylase Determination

| Specimen | pH* of Specimen | pH* of Incubation Mixture Initially | pH* of Incubation Mixture After Incubation | pH* after addition of Copper Reagent |
|---|---|---|---|---|
| Urine | 7.53 | 7.30 | — | — |
| Urine | 5.10 | 7.20 | — | — |
| Urine | 5.14 | 7.21 | — | — |
| Urine | 5.42 | 7.25 | — | — |
| Urine | 4.80 | 7.15 | 7.02 | 10.0 |
| Urine | 4.78 | 7.10 | 6.97 | 10.0 |
| Urine | 7.55 | 7.26 | 7.13 | 10.0 |
| Serum | — | 7.24 | 6.96 | 10.0 |
| Serum | — | 7.21 | 7.10 | 10.0 |
| Serum | — | 7.20 | 7.10 | 10.0 |
| Water** | — | — | — | 10.1 |

*All pH measurements were made at 25 °C.

**A mixture of 2 ml. of water and 1 ml. of Somogyi Copper reagent had a pH of 10.2.

in the presence of phosphate. Lineweaver-Burk plots of the two sets of data displayed identical slopes and intercepts, within the limits of experimental error. Finally, four batches of Hyland control sera having labeled activities between 600 and 1000 Somogyi units and four sera from patients without pancreatic disease were assayed using Substrates A and B. No significant differences were observed. Higher activities with Tris have been reported with amyloclastic (*14*) and turbidimetric (*15*) procedures.

The ability of zinc sulfate to stop the amylase reaction was confirmed by incubating 0.1-ml. aliquots of highly active control serum at 40° with 0.1 ml. of Tris-HCl buffer, *p*H 7.2, 1 ml. of Substrate C, and 2 ml. of 5% zinc sulfate for times ranging from 0 to 30 min. Deproteinization was completed and reducing sugars measured by methods described previously. No amylase activity was detectable.

### Selected Method

A routine saccharogenic amylase determination, evolved from foregoing experiments is summarized in stepwise fashion:

1. Pipet 1 ml. of Substrate C and 0.1 ml. of 1 M Tris-HCl buffer, *p*H 7.2, into four 15-ml. centrifuge tubes and place in a 40° water bath.
2. Add 0.1 ml. of water to the first tube (*RB*) and 0.1 ml. of 200 mg./100 ml. glucose standard to the second (*St*) and mix.
3. Add 0.1 ml. of serum or urine to the third tube (*X*), mix, and incubate all four tubes for 30 min. at 40°.

4. Add 2 ml. of 5% zinc sulfate to all 4 tubes and mix.

5. Add 0.1 ml. of serum or urine to the fourth tube ($C$) and mix.

6. Add 4.8 ml. of water and 2 ml. of 0.3 $N$ barium hydroxide to all tubes, shake vigorously, let stand for 10 min., and centrifuge for 10 min. at moderate speed.

7. Pipet 1 ml. each of supernatant, water, and Somogyi copper reagent into test tubes and mix.

8. Cover tubes with marbles and heat in an actively boiling water bath for 15 min.

9. After cooling tubes for 2 min. in a cold water bath, add 1 ml. of arsenomolybdic acid reagent to each tube, mix, let stand for 3 min., and transfer to spectrophotometer cuvets.

10. Measure absorbance of each at 540 m$\mu$ against water. When readings exceed 0.7, dilute with a measured amount of water, remeasure the absorbance, and multiply absorbance by dilution factor. Spectrophotometry should be carried out without unnecessary delay. About 3% darkening occurs between 3 and 15 min. after addition of arsenomolybdic acid reagent. Calculation is as follows.

$$\frac{A_X - A_C}{A_{St} - A_{RB}} \times 200 = \text{units amylase activity per 100 ml. serum or urine}$$

These units are equivalent to Somogyi saccharogenic units.

For accurate work, if a result of over 1400 units is obtained, repeat the entire determination using a more dilute enzyme solution. Serum or urine dilutions should be made with normal saline if the diluted specimens are to be stored. Otherwise, water may be used, since buffer contains sufficient chloride to permit the enzymatic reaction, to proceed at maximum velocity.

The preparation of a precalibrated glucose standard curve, run in the presence of substrate and deproteinizing reagents, is convenient and justified by the excellent day-to-day reproducibility of the quantity ($A_{St} - A_{RB}$). A control serum of known high amylase activity should be run frequently.

Independent duplicate analyses were performed with a precision of $\pm$ 11% at approximately 70 units per 100 ml. and $\pm$ 6% at approximately 200 units per 100 ml., at the 5% level of confidence.

## Discussion

In numerous papers on saccharogenic methods, the merits and disadvantages of various procedures for determining reducing groups

have been discussed with respect to reducing equivalence and reaction times required with different sugars. Possible inadequacies of these methods at high sugar concentrations have not been studied thoroughly, except in the case of a titrimetric copper reduction procedure (*16*). In addition to previously described experimental findings with a commercial amylase kit, several reports have appeared which arouse the suspicion that reducing sugars in amylase incubation mixtures are not always measured completely by the original and Tonks-modified Folin-Wu procedures.

In one series of 23 sera, subjected to parallel saccharogenic and amyloclastic assays which should have yielded roughly equivalent results, maximum values of 400 and 1070 units, respectively, were found (*17*). In two other series, in which the amylase activities of large population samples with known pancreatic disease were measured saccharogenically, the highest reported values were 372 and 440 Somogyi units (*18, 19*). The range of maximum values in those and the Krautman series, 300 to 440 units, corresponds well with plateaus in the Folin-Wu and Tonks curves shown in Fig. 2, allowing for preincubation concentrations of reducing sugars.

Enzyme inhibition, insufficient substrate, or sampling technics may all be possible causes for the low values observed in the series mentioned above. Inhibitors have now been shown to be absent from at least several batches of Krautman's substrate. Since Somogyi's incubation conditions provide a high enough ratio of starch to serum to permit maximum velocity to about 700 units (*1, 2*), insufficient substrate can be ruled out as a possible explanation for the low maximum values observed in three of the series (*17–19*). From the large size of the samplings of patients with known pancreatic disease (*7, 18, 19*), some higher values should have been anticipated. Furthermore a literature search failed to reveal any large study of pancreatitis patients exhibiting such low maximum amylase activities, when results were obtained by amyloclastic methods. Such discrepancies are too large to be explained by differences between Somogyi saccharogenic and amyloclastic units.

Extended-range amylase procedures, utilizing spectrophotometric copper reduction methods, have appeared previously. Teller (*11*) used a fivefold dilution of deproteinized incubation mixture to avoid cloudiness in the final colored solutions. Apparently he was unaware that, by diluting, he extended the range of his sugar determination fivefold, since he prepared still higher dilutions before carrying out

copper reductions on highly active specimens. The proposed method has several advantages over Teller's, including a somewhat more sensitive sugar quantitation, a higher starch-to-serum ratio, and better buffering. Teller's method has been criticized (*20*) because the deproteinized incubation mixtures are cloudy, and the degree of cloudiness differs markedly between incubated and control supernatants. Although this finding has been confirmed, it is not felt to be an important disadvantage, since cloudiness disappears from all solutions in the final dilution step. Furthermore, when Teller's procedure is modified by using a well-buffered (Tris or phosphate) substrate, the final colored solutions remain clear, although the turbidity of the deproteinized solutions is thereby increased (*21*).

A recently published micro procedure (*22*) has significantly extended limits of both incubation and sugar quantitation, but the latter is accompanied by a corresponding loss of sensitivity. The presence of sodium citrate during incubation may be another objectionable feature of that method (*23*). β-Amylase [sic] (*24*) activities in serum are claimed to be measurable with a single incubation and sugar determination to 5000 mg./100 ml. of glucose equivalent by the method of Friedman. This is surprising in view of the concentrations of substrate and copper employed. Andersch (*25*) and Natelson (*10*) have published methods which extend the limiting value measurable by single incubation and copper reduction to a point intermediate between that of the Somogyi (*2, 8*) or Henry and Chiamori (*1*) procedures and our present one.

An amylase result at least 4–6 times the upper limit of the normal range is important in distinguishing acute pancreatitis from acute abdominal diseases which might require emergency surgical treatment, and which are also capable of causing some elevation in amylase activity (*26*). Use of a procedure which requires repetition, totally or in part, to measure diagnostically significant values is unnecessarily time consuming. Use of a procedure which completely fails to measure such values renders the test virtually useless.

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# Barbiturate Detection using Thin-layer Chromatography

*Irving Sunshine, Eleanor Rose, and Jack LeBeau*

**Thin-layer chromatography has been applied to the analysis of barbituric acids. The ratio of the $R_f$ of 23 different barbituric acid samples to that of simultaneously run phenobarbital is given. This ratio is reproducible and characteristic. By using this technic the average laboratory can provide a barbiturate analysis of biologic material in about 2 hr.**

MEDICATIONS are one of the leading causes of fatal and nonfatal poisonings. Of all medications, salicylates (in children) and barbiturates (in adults) are most frequently involved in these poisonings. When these occur, the physician treating the victim may request a chemical analysis of a blood sample to confirm his clinical impression. A salicylate analysis is simple and rapid (*1*). However, a barbiturate determination is nonroutine, fairly elaborate, and beyond the facilities of most clinical laboratories (*2*). Thin-layer chromatography is suggested as a new technic that will permit the average laboratory to analyze biologic materials for barbiturates with relative ease and economy.

## Procedure

### Reagents

*Chloroform Acetone Mixture* 9:1 (v/v).

*$HgSO_4$ spray* Suspend, with mixing, 5 gm. of HgO in 100 ml. $H_2O$. Add, while mixing, 20 ml. conc. $H_2SO_4$. Cool, dilute to 250 ml. with $H_2O$.

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*Diphenylcarbazone spray* Dissolve 5 mg. in 50 ml. $CHCl_3$. Store in dark bottle.

*Fluorescein spray* To 500 ml. $H_2O$ add 20 gm. NaOH and 20 mg. sodium fluorescein.

*$KMnO_4$ spray* 0.1% aqueous solution.

*Standard barbiturate acids* 10 mg./100 ml. (w/v) in $CHCl_3$.

*Silica gel "G"* According to Stahl.

### Equipment

1. Standard apparatus for coating glass plates with a 250-$\mu$ layer of silica gel "G"
2. Rectangular jars, $8\frac{1}{2} \times 4 \times 8\frac{1}{2}$ in. with ground-glass cover
3. Spray bottles
4. Tuberculin syringes with size 24 ¼-in. needles
5. Mineralite ultraviolet hand lamp with 254 m$\mu$ filter

## Method

Plates (20 × 20 cm.) were coated with Silica gel G using standard thin-layer chromatography technics (*3*). These coated plates were dried in a ventilated oven for 20 min. at 100°. The dried plates were then stored at room temperature for subsequent use. Prior to an analysis, lines ½ in. apart were ruled on the plate, forming a series of parallel columns for concurrent analyses of many samples. Two drops of a chloroform solution of each sample were applied at a point 1 in. from the bottom of the plate in the center of a column. The spots were air dried and then the plate was placed in the rectangular jar containing 110 ml. of the 9:1 chloroform-acetone mixture. The mixture should be placed in the jar at the beginning of the preparation for this analysis to insure saturating the jar with the mixture's vapor. This jar can be reused and its solvent mixture need not be changed for 2 days.

In this solvent system, the solvent front will rise 10–12 cm. in 45–60 min. The plate is then removed and the solvent front marked immediately. Then the plate is air dried at room temperature. When dry, the plate is sprayed first with the $HgSO_4$ (*4*), allowed to dry partially, and then sprayed again with the diphenylcarbazone. In each instance the size, position, and color intensity of each spot is noted. An excess of the diphenylcarbazone is used. The overall colored background resulting slowly disappears and purple or violet spots remain to allow accurate determination of their respective $R_f$ values.

A second identification system that has proved effective consists of an initial spray with alkaline fluorescein. When viewed under 254 mμ light the barbiturates give a purple spot against a yellow-green background. After completing this observation, the plate may then be sprayed with the $KMnO_4$ (*5*). All unsaturated substituted barbiturates yield yellow spots against a purple background. The same $KMnO_4$ spray can be used without the fluorescein to give similar data.

Since 12–14 columns can easily be ruled on one plate, the same sample and suitable standards can be applied to consecutive columns, and then a second or third series can be placed on the next group of columns on the same plate. Several different sprays can then be used on one plate by suitably masking all but one series of columns for each spray. On each plate, known barbiturates, singly and in mixtures, should be run.

### Sample Preparation

Samples for analysis are isolated by direct $CHCl_3$ extraction at *p*H 7. Blood and tissue samples require no *p*H adjustment, but urine, stomach content, and drugs should be made alkaline or dissolved in NaOH. The alkaline solution should be filtered and a 3–5 ml. sample taken. These are then made mildly acid with dilute mineral acid and extracted with 50 ml. $CHCl_3$.

A filtered 40-ml. aliquot of the chloroform is then evaporated to dryness under forced air and mild heat. The residue is dissolved in 0.5 ml. $CHCl_3$ and portions of this solution are applied to the plate along with suitable standards and mixtures thereof.

## Results and Discussion

Data are presented for 23 barbiturates in Table 1. The absolute $R_f$ values were not recorded because these vary from one run to the next. However, when the ratio of the $R_f$ of any sample to that of phenobarbital is determined, these ratios are reproducible and significant. Those given in Table 1 represent the average of 15–20 determinations, each run on different days. Ninety per cent of these determinations gave $R_f$ ratios that were within ±0.10 of these reported in Table 1.

The $R_f$ ratio is enough to indicate the relative activity of the barbiturates from 1.0 for those of long activity to over 2.0 for the very short-acting. Additional data from the reaction to the spray reagents allow more specific identification, although for clinical purposes this is

**Table 1.** $R_f$ Ratios of Barbituric Acid to Phenobarbital (Silica Gel G Solvent: Chloroform-Acetone, 9:1)

| | | | *Sprays** | | |
|---|---|---|---|---|---|
| *$R_f$ ratio* | | *Drug* | *HgO* | *DPC* | *$KMnO_4$* |
| 1.00 | | Phenobarbital | W | V | |
| 1.00 | | Barbital | W | V | |
| 1.19 | U† | Vinbarbital | W | V | Y |
| 1.28 | | Butabarbital | W | V | |
| 1.30 | A† | Allobarbital | None | B | Y |
| 1.30 | A | Aprobarbital | None | B | Y |
| 1.32 | U | Heptabarbital | W | V | Y |
| 1.38 | U-A | Cyclopal | None | B | Y |
| 1.41 | | Butethal | W | V | |
| 1.41 | A | Talbutal | W | B | Y |
| 1.42 | | Pentobarbital | W | V | |
| 1.44 | | Amobarbital | W | V | |
| 1.44 | A | Allylbarbituric acid | W | B | Y |
| 1.50 | A | Butallyonal | W | B | Y |
| 1.56 | | Hexethal | W | V | |
| 1.67 | A | Secobarbital | W | B | Y |
| 1.77 | A | Sigmodal | W | V | Y |
| 2.06 | U | Hexobarbital‡ | None | V | Y |
| 2.35 | | Metharbital‡ | None | V | |
| 2.37 | | Mephabarbital‡ | W | V | |
| § | | Thiopental‖ | W | | Y |
| § | A | Thiamylal‖ | W | B | Y |
| § | U | Cyclobarbital | W | B | Y |

*B indicates blue; V, violet; Y, yellow; W, white.
†A indicates allyl substituent present in 5 position; U, unsaturated side chain present in 5' position.
‡N methyl barbiturates.
§Run with solvent front.
‖Thiobarbiturates.

not essential. The yellow spots following a $KMnO_4$ spray identified all thio-barbiturates as well as those with unsaturated substituents. The diphenylcarbazone spray gives a dark-blue color with those barbiturates containing an allyl group and with cyclobarbital. All these spots are also semiquantitative and comparison with standards permits an educated guess as to their concentration.

In addition to pure drugs this thin-layer technic has been successfully applied to biologic samples from over 100 victims of barbiturate intoxication. In 90–120 min. after starting the extraction, one could report whether or not one or more barbiturates were present, which drugs were probably implicated, and an educated guess as to how much

was present. This is a reasonable answer to the question "was a barbiturate ingested by my patient?" Most important, it is an answer which can be given by any laboratory which has the simple and inexpensive equipment required. This involves only a $300 investment compared to the $3000 required for a more precise answer based on ultraviolet spectrophotometry. It has an advantage over the spectrophotometric procedure in that one can easily ascertain whether more than one barbiturate is involved.

Unfortunately it does not permit a precise quantitative estimation, but this is a small price to pay for a heretofore unavailable determination.

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# Rapid Cellulose Acetate Electrophoresis

## I. Serum Proteins

*Raymond C. Bartlett*

**A method of electrophoresis utilizing cellulose acetate is described wherein quantitation of serum protein fractions by densitometry is accomplished within 2 hr. of initiation of the procedure. The densitometer was found to have a linear response to amido black dye. Albumin and γ-globulin demonstrated equal dye-binding characteristics. Combined with the absence of albumin trailing, this has afforded excellent quantitations of serum protein fractions.**

PAPER ELECTROPHORETIC ANALYSIS of the major serum proteins has become firmly established in clinical laboratories. The complexity of the processes involved and shortcomings in available technics have led to numerous modifications in apparatus and procedure. The result of this has been a marked lack of standardization between laboratories (*1*). Further complication has resulted from the introduction of supporting media such as starch (*2–4*), agar, and acrylamide gels (*5–6*) capable of yielding considerably finer fractionation of proteins than can be accomplished on filter paper. Immunoelectrophoresis has afforded the greatest qualitative sensitivity. The clinical utility of these newer methods must await the correlation of the data obtained with disease states.

We have performed over 5000 serum protein paper electrophoretograms using the Durrum procedure.* Inadequacies of this technic have been observed by us and are well documented by others (*14–16*). These include excessive time required, albumin trailing, and inconsistent reproducibility. Determination of abnormal hemoglobins and quantitation of hemoglobin A2 has required additional apparatus. This report

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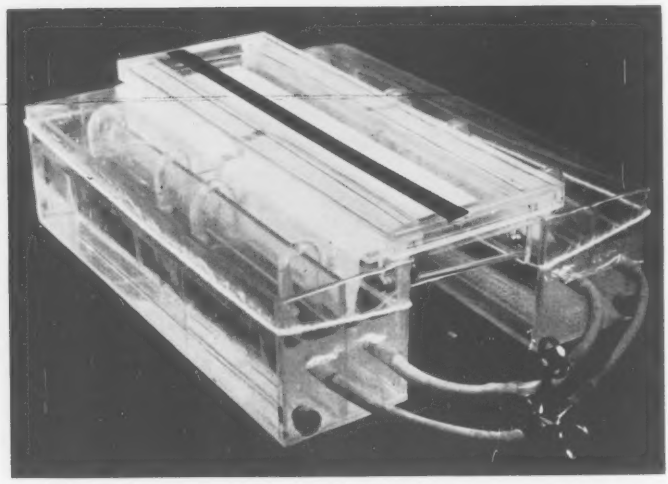

**Fig. 1.** Apparatus constructed for cellulose acetate electrophoresis.

describes the evaluation of a technic which has been developed to obviate these difficulties. Cellulose acetate was adopted as the supporting medium. Other workers have established the superiority of this medium over filter paper (*17–24*).

## Materials

1. Tanks suitable for cellulose acetate electrophoresis; these are commercially available.* Our apparatus was constructed in the laboratory and is depicted in Fig. 1 and 2.

2. Any power supply capable of producing constant current up to 50 ma at 200–300 v is suitable.

3. Cellulose acetate strips, $5 \times 36$ cm.†

4. Barbiturate buffer, 0.10 M, *p*H 8.6: sodium diethylbarbiturate, 73.0 gm.; diethyl barbituric acid, 9.0 gm.; water, 4000 ml. Dilute to 0.03 M for tanks and impregnation of strips.

5. Amido black stain: amido black (Naphthol blue black), 10 gm.; methyl alcohol, 400 ml.; distilled $H_2O$, 400 ml.; glacial acetic acid, 100 ml.

6. Washing solvent: methyl alcohol, 600 ml.; distilled water, 200 ml.; glacial acetic acid, 100 ml.

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†Shandon; Consolidated Laboratories, Chicago Heights, Ill.

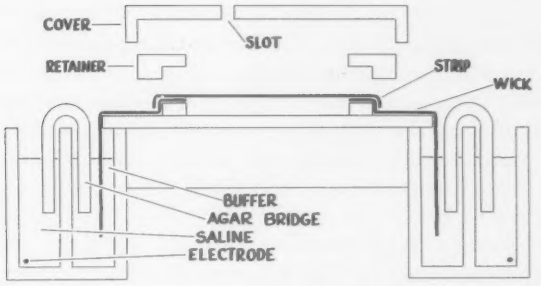


**Fig. 2.** Diagram of apparatus showing arrangement of buffer tanks, filter paper wicks, and position of electrophoretic strip.

7. The Model RB Analytrol* automatic recording densitometer equipped with 500 m$\mu$ interference filters and a B5 cam was used.

## Procedure

Cellulose acetate sheets are cut into strips 2.5 × 12 cm. in size, labeled, and saturated with buffer. After blotting, strips are placed in the apparatus, whereupon 3 $\mu$l. of serum are applied (transversely across the center of the strip) either by a stripper or with a capillary tube. Constant current of 0.4–0.5 ma/cm. width of strip is applied. This will result in an initial potential of about 150–200 v at the strip ends. After an hour, the strips are removed, dried at 100°–105° for 10 min., placed in amido black dye for 5 min., and transferred to the solvent bath. After 15 min., the background is white and they are dried between filter paper sheets.

Densitometry is accomplished by placing the strips in a transparent sleeve which is run through the Analytrol densitometer.

### Glycoproteins and Lipoproteins

The periodic acid-Schiff reaction staining technics, as described in the original papers, may be used for both glycoproteins (*25*) and lipoproteins (*26*). Minimal background coloration is observed with these methods.

## Discussion

A buffer was selected for this procedure that would be of sufficiently low ionic strength to afford rapid migration but with enough buffering capacity to prevent distortion; 0.03 M barbiturate buffer was found optimal. No insolubility of macroglobulins was found at this molarity

*Spinco Division, Beckman Instruments Company.

as reported by Ressler *et al.* (*27*). Constant current was selected because this prevents excessive heating by reducing voltage as resistance decreases as a result of evaporation.

Sunderman suggested standardizing heat denaturation at 100°–105° for 30 min. on filter paper (*1*); 10 min. at this temperature was found adequate for cellulose acetate. Bromphenol blue, amido black, ponceau S (*13*), lissamine green (*23*), and other stains have been described for use with cellulose acetate. We desired a stain that could be used with our recording densitometer without requiring extensive alteration of the instrument. The B5 compensating cam provided with

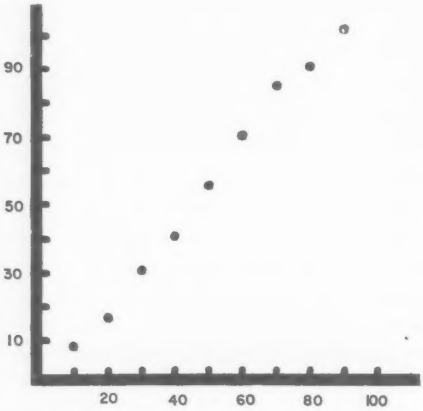


**Fig. 3.** Absorbance of Amido black dye on cellulose acetate. Integration units plotted on ordinate; percentage dye concentration on abscissa. (Spinco Analytrol; 500 mμ interference filters.)

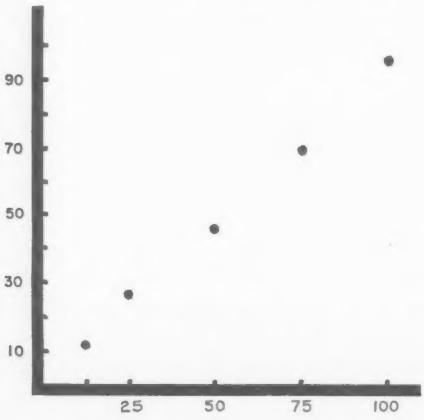


**Fig. 4.** Absorbance of stained serum dilutions. Integration units plotted on ordinate; percentage serum concentration on abscissa.

our instrument is calibrated to give a linear response to bromphenol blue dye on filter paper. Amido black gives a more permanent and intense staining of cellulose acetate strips and a more equal affinity for binding of albumin and globulins has been described (*28*). No increase in protein binding after 5 min. of staining time was observed.

Linearity of the instrument with the B5 cam and 500 mμ filters was demonstrated for amido black dye applied in increasing concentration to cellulose acetate strips (Fig. 3).

Dilutions of pooled serum were applied to strips which were heat-denatured and stained. Densitometry yielded a linear response throughout the working range (Fig. 4).

Electrophoretically pure solutions of albumin and γ-globulin were prepared. The final concentration was determined by micro Kjeldahl analysis and found to be 4.50 gm./100 ml. for albumin and 4.35 gm./100

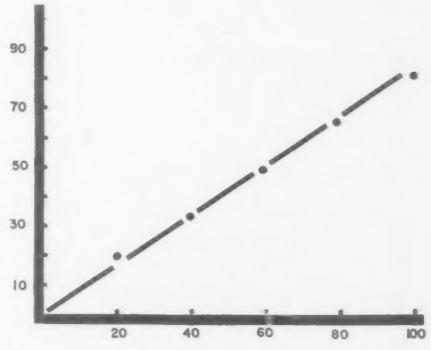


**Fig. 5.** Absorbance of stained dilutions of albumin. Integration units plotted on ordinate; percentage concentration on abscissa.

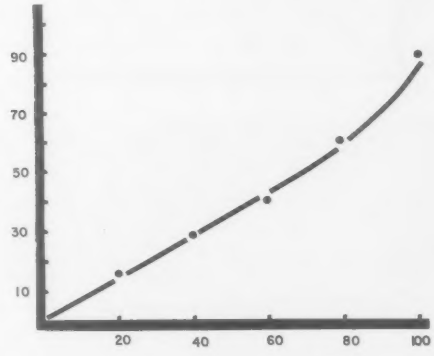


**Fig. 6.** Absorbance of stained dilutions of γ-globulin. Integration units plotted on ordinate; percentage concentration on abscissa.

ml. for $\gamma$-globulin. These solutions were applied in increasing dilutions to cellulose acetate strips which were heat-denatured and stained. Densitometry demonstrated linearity with concentrations up to 4.5 gm./100 ml. of albumin (Fig. 5). Nonlinearity was observed when $\gamma$-globulin concentration exceeded 3.5 gm./100 ml. (Fig. 6). Solutions of albumin and $\gamma$-globulin consisting of 50, 60, 70, 80 and 90% albumin were prepared and subjected to electrophoretic separation. Units of integration from the densitometer were converted into percentages which were corrected for the absolute concentrations of the solutions. The electrophoretic percentage was plotted against the predetermined percentages based on chemical analysis. Excellent agreement was found (Fig. 7).

The reproducibility was determined by 10 replicate analyses of a serum sample. The coefficient of variation is seen to be comparable to that for filter paper electrophoresis as reported by Sunderman (*1*) (Table 1).

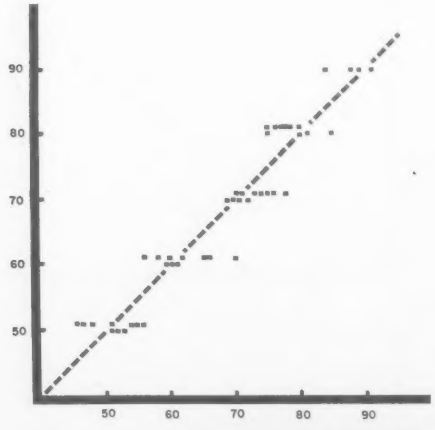


**Fig. 7.** Agreement between percentage concentrations of albumin over $\gamma$-globulin as determined chemically (ordinate) and electrophoretically (abscissa).

**Fig. 8.** Appearance of stained serum protein electrophoretic strip. Point of application at center, albumin at left, $\gamma$-globulin at right.

**Table 1.** Comparison of Reproducibility with Electrophoresis (C.V.)

| | *Alb.* | *Alpha₁* | *Alpha₂* | *Beta* | *Gamma* |
|---|---|---|---|---|---|
| Cellulose acetate (n = 10) | 8.6% | 14.8% | 9.0% | 9.0% | 15.1% |
| Filter paper (n = 8) | 7.0% | 16.2% | 11.3% | 11.4% | 7.3% |

**Table 2.** Range of Serum Protein Fractions from 26 Blood Donors

| *Alb.* | *Alpha₁* | *Alpha₂* | *Beta* | *Gamma* |
|---|---|---|---|---|
| 64% (45–75%) | 3.8% (2–6%) | 10.3% (6–15%) | 8.8% (5–12%) | 13% (6–21%) |

The range for apparently normal individuals was estimated by analysis of 25 serum specimens from randomly chosen blood donors (Table 2). The absence of albumin trailing is reflected in albumin values higher than generally observed on filter paper.

## Addendum

Further development of this technic has demonstrated that comparable results are obtained with an inexpensive commercially available electrophoresis tank.* The only change in the method has been an increase in the buffer strength to 0.05 M.

Since completion of this manuscript, several reports have been published concerning cellulose acetate electrophoresis. Afonso (*29*) stated that sharper separations were achieved using higher voltage for shorter times with addition of glycerol to the buffer to reduce evaporation. Ohlsen and Charlton (*30*) and Friedman (*31*) reported general methods along with mean normal values. Rubinstein *et al.* (*32*) compared electrophoresis of proteins from normal and abnormal sera in cellulose acetate and starch gel. Grunbaum (*33*) demonstrated densitometry of cellulose acetate strips with the Spinco Analytrol using Ponceau S stain.

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# Rapid Cellulose Acetate Electrophoresis

## II. Qualitative and Quantitative Hemoglobin Fractionation

*Raymond C. Bartlett*

**A method is described for the qualitative and quantitative analysis of abnormal hemoglobins including hemoglobin $A_2$. Quantitative values are comparable to those observed with the starch block technic. The analysis is completed within 2 hr. The apparatus is the same as that used for quantitative analysis of serum proteins.**

IN ANOTHER REPORT, we have given a description and evaluation of a procedure for the rapid separation of serum proteins on cellulose acetate (*1*). One of the disadvantages of the methods in use previous to cellulose acetate was the necessity to maintain three different types of equipment for the separation of serum proteins, abnormal hemoglobins, and quantitation of hemoglobin $A_2$. In this report the use of the same apparatus and general procedure previously described for separation of serum proteins is applied to the identification and quantitation of hemoglobins.

### Qualitative Separation of Hemoglobins

#### Materials

Apparatus and electrical equipment has been previously described (*1*). Additional material required is Tris-EDTA-borate buffer, 0.06 M, *p*H 8.6 (Tris(hydroxymethyl)aminomethane (TRIS) Sigma 121, 24.2 gm.; disodium ethylenediaminetetra-acetate, 3.13 gm.; boric acid, 1.84 gm.; and distilled water, 4000 ml.).

#### Procedure

Hemoglobin solutions are prepared by the technic of Chernoff (*2*), in which hemolysis of red blood cells with distilled water is followed by

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shaking with toluene and by high-speed centrifugation. Aqueous solutions of hemoglobin are stored at 4°. The buffer chambers of the apparatus are filled with 0.06 M Tris-EDTA Borate buffer. This buffer is also used to saturate the strips, after which they are blotted between filter paper to remove excess surface moisture. Although 2.5 × 12-cm. strips, as described for serum protein electrophoresis, may be used, more samples may be simultaneously compared on the same strip if 5 × 12-cm. strips are used. One-microliter samples of the 10% hemoglobin solution are applied at 1-cm. intervals along a transverse midline which is drawn across the strip prior to saturation with buffer. Constant current at 0.4–0.5 ma/cm. width is applied resulting in an initial potential at the strip ends of 250–300 v. Separation is usually well defined in 40 min., whereas maximum separation occurs in 1 hr. Hemoglobins C, S, and A are clearly differentiated (Fig. 1). Normal amounts of $A_2$ are just visible as a tiny spot when a strong light is projected through the strip. When $A_2$ is present in amounts exceeding 4% the spot is clearly visible without the use of transmitted light. This observation allows elevated $A_2$ values to be anticipated in screening hemoglobin solutions.

## Quantitation of Hemoglobins

Unlike the case with filter paper, cellulose acetate is capable of maintaining the resolution of quite large amounts of protein applied for separation. Subsequent elution of the separated fractions allows quantitation by measurement of the absorbance in the Soret band by spectrophotometry. The amount of hemoglobin solution applied for quantitation is greatest when one of the fractions to be measured is very small. Hence quantitation of hemoglobins C, S, and A may be accomplished by applying about 5 $\mu$l., whereas quantitation of hemoglobin $A_2$ is achieved by applying 15–20 $\mu$l.

### Procedure

The procedure described for qualitative separation is followed except that one 5 × 12-cm. strip is used for each separation performed. The total sample (5 or 15–20 $\mu$l.) is divided among 4 or 5 spots equally placed across the transverse midline of the strip. Electrophoresis is conducted for about 40 min. at which time a clear zone is present between fractions. The strip is removed and cut transversely between fractions. Each transverse strip is placed in tubes containing Tris

EDTA Borate buffer. When measuring hemoglobins S, C, and A each is eluted into a volume of 3 cc. of buffer. In the case of hemoglobin $A_2$ quantitation, the A fraction is eluted into 9 cc. of buffer and the $A_2$ fraction into 3 cc. of buffer. Elution is complete in 20 min. The solu-

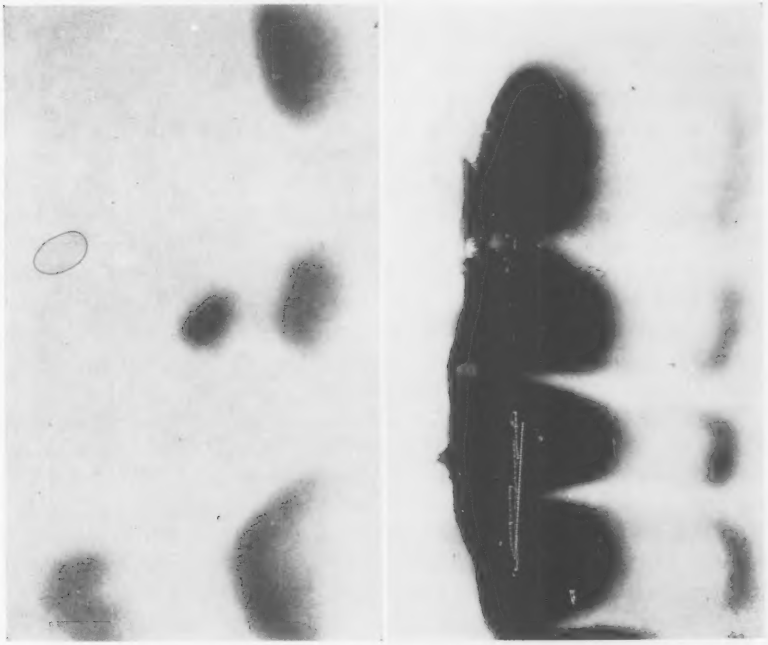

**Fig. 1.** (*left*) Qualitative separation of hemoglobins; top to bottom, A, SA, CA.
**Fig. 2.** (*right*) Quantitative separation of hemoglobins: right, $A_2$ (4.2%); left, A.

tions are measured in suitable cuvettes in a spectrophotometer at a wave length of 400–410 m$\mu$ in the Soret band. Percentages are then calculated.

## Discussion

Cellulose acetate demonstrates several features making it superior to filter paper for hemoglobin separation. On the other hand it shares the simplicity and ease of manipulation not afforded by other media such as starch, agar, and acrylamide gels. The absence of adsorption yields clean white backgrounds between fractions and there is no ad-

sorptive loss of protein during migration. The electroendosmotic flow is greater than in filter paper. This results in a continuous flow of buffer toward the cathode. Proteins having no net charge will migrate toward the cathode under these conditions. Furthermore, proteins whose net charge does not result in a velocity of migration toward the anode which is as great as the velocity of the endosmotic flow will appear as migrating cathodally. On paper, hemoglobin A migrates further toward the anode from the application line than hemoglobins $A_2$, S, and C. Therefore, hemoglobin A always traverses the site of final location of these other fractions. Adsorption and diffusion lead to lack of clarity in the separation. On cellulose acetate, A hemoglobin remains at or near the site of application whereas $A_2$, S, and C are carried toward the cathode by the endosmotic flow. This allows these fractions to separate from A hemoglobin under conditions much more favorable for clear separation than on paper. Methods for the separation of hemoglobins A and $A_2$ have been described using filter paper (*3–5*). Marked overlapping of the two fractions occurs with these technics. It will be seen from Fig. 2 that a perfectly sharp zone of separation is produced on cellulose acetate.

The normal range of hemoglobin $A_2$ by starch block technic has been stated by Gerald and Diamond to be 2.54% ± 0.35% (*6*). In thalassemia minor these authors found levels of 5.11 ± 1.36%. The error for the starch block method is stated to be ±0.09% — ±0.11%.

The normal range for this hemoglobin measured by the cellulose acetate technic was determined for 17 random normal hemoglobin specimens and found to be 2.5% ± 0.7%. The error (± 2 S.D.) was ± 1% based on 10 replicate analyses of a sample showing a mean $A_2$ value of 4%. Cases of thalassemia minor thus far studied have shown values between 4% and 6%. Two instances were discovered on routine qualitative separation when the prominence of the small $A_2$ spot suggested that this fraction might be elevated. The simplicity of the technic allows duplicates to be performed, thereby greatly reducing the analytical error.

## Addendum

Additional development of this technic has demonstrated that comparable results are achieved using an inexpensive commercially available electrophoretic tank.* An increase in buffer strength to 0.10 M

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gave optimal results. The author wishes also to acknowledge a recent report by Afonso (*7*) in which a reverse-flow technic was applied to cellulose acetate membranes for successful separation of hemoglobins A and $A_2$.

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# A Simple and Rapid Method for the Paper Electrophoretic Determination of Urinary Proteins

*Richard Collens, Harry Meyers, and Kurt Lange*

**A method is described for the simple, rapid, and accurate determination of paper electrophoretic patterns of urinary proteins using polyethylene glycol (Carbowax 20 M) for the concentration of the urine. The electrophoretic patterns of sera with normal and abnormal protein constituents did not change when added to a protein-free urine and subjected to the method described. The electrophoretic determination of urinary proteins as a routine procedure may give an insight into the physical type of permeability disturbance in the kidneys of a patient.**

A NUMBER OF ATTEMPTS have been made to determine urinary proteins by electrophoresis, in diseases with renal leakage of normal or abnormal serum proteins (*1–5*). It appears, however, that none of the methods used in these determinations were simple enough to permit paper electrophoresis of urinary proteins as a routine procedure. The advantages of performing such determinations on a large scale in order to estimate the permeability defect of the glomerular membrane are obvious. It remains to be seen whether these defects differ in different renal diseases or stages of diseases and whether or not a typical pattern can be correlated with such conditions. Hardwicke and Soothill (*4*) believe that certain leakage patterns can be related to certain specific morphologic alterations (e.g., membranous glomerulonephritis).

It must be remembered in this connection that a proteinuria of identical magnitude can be caused by a large number of mildly protein-

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leaking nephrons on one hand or by a small number of nephrons with severe protein leakage on the other. In such circumstances, however, the ratio between proteins of a relatively small size (e.g., albumin) and those of a large size (e.g., gamma globulin) would differ considerably. In addition, the question of loss of antibody gamma globulin into the urine in certain renal diseases can be approximated if electrophoretic determinations of urinary proteins are done routinely. It appeared therefore important to develop a method for the simple, rapid, and inexpensive electrophoretic determination of urinary protein.

## Procedure

The urine specimens are centrifuged for 30 min. at 2000 r.p.m., decanted, and filtered through Whatman No. 40 paper. The protein concentration of each specimen is determined by the Biuret method (*6*). Calculations are then made to determine the concentration necessary to bring the protein in the urine to approximately 5 gm./100 ml., which is needed to obtain a distinct paper electrophoretic pattern. The initial volume of urine is selected so as to give a final volume of approximately 1 ml. after concentration. This amount is then encased in a bag of Visking* cellulose dialysis tubing which is then suspended in a bath of 40% polyethylene glycol (Carbowax† 20 M, mol. wt. 20,000) dissolved in Veronal‡ buffer, *p*H 8.6 and ionic strength 0.075. The bath is agitated continuously by a magnetic stirrer at 4°. In approximately 5 hr., the necessary concentration of the urine is reached and the specimen can be applied to the paper strip. Occasionally a cloudy precipitate appears in the urine after concentration. This precipitate can be removed by centrifugatioin for 5 min. at 3000 r.p.m. It does not contain protein and its removal does not affect the electrophoretic pattern.

The Beckman-Spinco Durrum§ cell was used for the present electrophoresis studies and the Beckman RB Analytrol§ for the quantitative analysis of the paper strip after staining with alcoholic bromphenol blue.

In order to see whether the procedure itself alters the protein pattern in the urine, 0.5 ml. of a serum with 8.5 gm./100 ml. of proteins which contained an abnormal myeloma protein was added to 9.5 ml. of a urine which by itself showed no electrophoretic mobility when con-

*Visking Corp., Chicago, Ill., seamless dialyzer tubing 1⅛ in. wide.
†Union Carbide Chemical Co., Charleston, W. Va.
‡15.4 g. sodium barbital, 2.76 g. barbituric acid to 1000 ml. $H_2O$.
§Beckman Instruments, Inc., Palo Alto, Calif.

centrated 10 times. This prepared sample was handled as described above and subjected to paper electrophoresis at the same time as the original serum. The results are shown in Table 1. The table indicates that the procedure itself does not introduce an error beyond the inherent error of the paper electrophoretic method. Other experiments using sera containing normal and abnormal proteins, and normal and abnormal concentrations of proteins again did not show any significant deviation from the type of results shown in Table 1.

The dialysis of the urine against polyethylene glycol (Carbowax 20 M) dissolved in distilled water did not yield different results from the dialysis against polyethylene glycol dissolved in the Veronal buffer. It was felt, however, that the latter method was preferable to maintain a uniform buffer system throughout the procedure. Carbowax 6000 (mol. wt. 6000) diffuses through the membrane of Visking tubing, as can be shown by precipitation with 20% trichloroacetic acid. We were able to show, as previously reported by Tombs *et al.* (*7*) that the diffusing polyethylene glycol did not alter the electrophoretic pattern of the urinary protein. It was felt, nevertheless, that the use of a nondiffusing Carbowax would be preferable. Therefore, we used Carbowax 20 M (mol. wt. 20,000) in our studies, which does not diffuse through the membrane of the Visking tubing used in our laboratory. It thus appears that this method does not lead in itself to any changes in the protein pattern.

Most recently, Hardwicke and Soothill (*4*) and Slater and McKay (*8*) have shown with immunochemical methods that normal urine may contain a gamma globulin of a very small molecular size, possibly derived directly from tubular protein breakdown. While the presence of such a protein will require further confirmation, it is obvious that such a moiety of small molecular size would not appear in a paper electrophoretic pattern since it might be lost into the bath fluid during the concentration process. Since urinary protein determinations will serve

**Table 1.** RESULTS OF ELECTROPHORESIS BY USING POLYETHYLENE GLYCOL FOR CONCENTRATION OF URINE

| *Protein fraction* | *Original serum (% of total protein)* | *Urine plus serum (% of total protein)* |
|---|---|---|
| Albumin | 26.3 | 25.2 |
| Alpha-1 globulin | 3.0 | 3.5 |
| Alpha-2 globulin | 4.5 | 4.9 |
| Beta globulin | 6.8 | 7.0 |
| Gamma globulin | 59.4 | 59.4 |

essentially to determine plasma protein clearances and the permeability of the glomerular membrane, the loss into the urine of proteins of such small molecular weight, which do not appear in the plasma protein pattern, may be considered an advantage rather than a disadvantage.

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# Enzymatic Oxalate Determination in Urine

*Gerda G. Mayer, Deborah Markow, and Frieda Karp*

**A method depending upon the use of oxalic decarboxylase is described for the determination of oxalic acid in urine. The urinary oxalate excretion of 25 healthy persons measured by this procedure is found to average 20.5 mg. $(COOH)_2$/24 hr. In 70 patients with calculus disease the oxalate titer of the urine was within the normal range.**

THE ACCURATE MEASUREMENT of oxalate concentration in urine presents considerable difficulties. The methods available in the literature are based upon either the direct formation of calcium oxalate precipitate and subsequent oxidimetric titration (*1–3*) or upon a preliminary ether extraction followed by a titrimetric procedure (*4, 5*). In a more elaborate method, the extraction with ether is combined with a colorimetric determination (*6*). Perhaps the most reliable approach devised to date employs radioactive-labeled oxalic acid (*7*), but this procedure involves special equipment not available in many laboratories.

This report describes a rapid method for the determination of oxalic acid in urine utilizing the oxalic decarboxylase from the wood-rot fungus *Collybia velutipes* and originally suggested by Dr. H. Shimazono. Oxalic acid is stoichiometrically converted to formic acid and $CO_2$ according to the reaction (*8*):

$$\begin{matrix} COOH \\ | \\ COOH \end{matrix} \longrightarrow HCOOH + CO_2$$

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The authors wish to thank Dr. Hans H. Zinsser for his encouragement on this project and also Dr. W. B. Jakoby for his help and valuable suggestions at the outset of our work. We are most grateful to Dr. Harry Seneca and Dr. Margarita Silva for their help in handling the *Collybia velutipes*.

The chief advantage of this procedure lies in its complete specificity for oxalic acid. Recently it has been applied by Crawhall (*9*) and Haas (*10*) to assay oxalic acid in plasma and in beer. In the present paper we show that this enzyme may be used for accurate measurement of oxalic acid in urine.

## Materials and Methods

### Enzyme

Mycelia of *Collybia velutipes* (Strain S*) (*11*) is prepared as described by Shimazono and Hayaishi (*8*) and the extract is purified through the dialyzate of their "first acetone fraction." The enzyme solution may be stored at −15° in 0.02 M potassium acetate buffer at *p*H 4.5 for a maximum of 3 weeks with no noticeable loss of activity. The enzyme preparations were assayed and an amount was used sufficient for complete decarboxylation of 10 $\mu$M of potassium oxalate within approximately 30 min. Six different preparations of the oxalic decarboxylase gave activities ranging from 2.7 to 4.2 U./ml., when the unit is defined as that amount which catalyzes the formation of 1.0 $\mu$M of $CO_2$ per minute employing the "standard assay" procedure as described below. These activities were adequate for the analysis of urine specimens with a wide range of oxalate concentrations. The course of the reaction when 10 $\mu$M of potassium oxalate was placed in the Warburg flasks is shown in Fig. 1.

### Treatment of Urine and Preliminary Stages

The 24-hr. collection of urine was preserved under toluene. However, regardless of how the urine is collected and stored, it has not been possible to prevent a moderate increase in oxalate detectable after urine was stored. The production of oxalate in whole urine is diminished but not completely prevented by storage of the specimens at 4°, and even a temperature of −15° is found insufficient to prevent the change completely. Addition of up to 5 ml. of concentrated HCl per 1000 ml. is also ineffective. The amount of oxalate formed on standing in the cold is small but appreciable; therefore for accurate estimation of urinary oxalic acid excretion it is desirable that the specimens be assayed as soon as possible after collection.

The procedure used in our laboratory was as follows. The oxalic acid in a 50-ml. aliquot of a 24-hr. collection of urine was precipitated

*Obtained as A.T.C.C. 13547 from the American Type Culture Collection, Washington 7, D. C.

at a *p*H of 5.0–5.2 by the addition of 2.0 ml. of 5% (w/v) $CaCl_2$. After this addition, the urine was usually allowed to stand a minimum of 3 hr. at room temperature and was then kept overnight at 4°. The sample was centrifuged at 2400 rpm for 30 min. at 0°. The supernatant

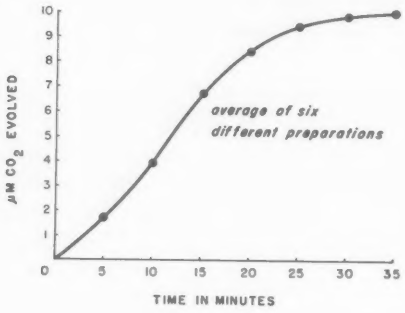


**Fig. 1.** Rate of oxalic acid decarboxylation using 0.1 ml. enzyme solution.

was carefully decanted and allowed to drain. The precipitate need not be washed. Losses by washing which were shown to be appreciable are thus avoided. The precipitate was dissolved in 10 ml. of 0.1 M potassium citrate buffer *p*H 3.2. This procedure required at least 1 hr., with gentle heating of the suspended precipitate in a water bath at 70–80°. The volume was then readjusted to 10 ml. with water.

### Assay

A 2.0-ml. aliquot of the 10-ml. solution is introduced into a Warburg flask containing 400 μM potassium citrate (*p*H 3.0), 6 μM ethylenedinitrilotetraacetic acid disodium salt (EDTA), and 0.1 ml. of enzyme preparation (side arm) in a total volume of 3.0 ml.

Each assay consists of the sample, a standard (10 μM of potassium oxalate, *p*H 3.0), and a flask containing both sample and standard. The third flask is used for evaluation of a recovery of added oxalate and can be omitted once an average recovery rate has been established. Each assay is run in duplicate.

The reaction flasks are kept in ice until they are attached to the manometer and put in the bath at 37°. They are equilibrated for 10 min. open to the atmosphere. The manometers are then closed and equilibration continued for 5 more min., when the reaction is started by tipping the enzyme into the vessel. Readings are taken every 5 min. until the reaction is completed, usually within 1 hr.

The concentration of oxalic acid in the urine is calculated as follows.

$$\frac{\text{mg. } (COOH)_2}{\text{24 hr.}} = \frac{\mu M\ CO_2 \text{ generated} \times \text{M.W. } (COOH)_2 \times \text{total vol./24 hr.}}{10 \times \text{recovery} \times \text{volume of aliquot}}$$

## Results

Although the specificity of this enzyme has been demonstrated for serum (*9*) and beer (*10*), no studies have been made of its accuracy for analysis of urine. Therefore, tests were carried out using substances commonly found in urine in a quantity possibly sufficient to interfere with this assay. As we have reported previously (*12*), glucuronic acid has been a major concern.

Sodium glucuronate was investigated because of its co-precipitation with oxalic acid in urine upon the addition of calcium ions (*13*). When chromatographically pure sodium glucuronate was assayed alone and with the oxalic acid standard in concentrations up to 50 $\mu$M in 3 ml., the enzymatic oxalic acid assays were unaffected by the presence of glucuronate.

Uric acid was tested because of its poor solubility at *p*H 5.2. The addition of as much as 15 mg. of uric acid did not inhibit the rate of decarboxylation of the standard 10 $\mu$M of potassium oxalate nor did it depress the production of $CO_2$ in the samples so treated. Cystine in amounts up to 12 mg. assayed under similar conditions did not interfere with the enzymatic determination. These compounds were tested because of their interference in the permanganate titration.

Phosphates are carried down with calcium oxalate precipitates. The addition of 30 mg. of phosphate in the form of $KH_2PO_4$ inhibited the rate of reaction but permitted it to go to completion after 1½ hr. Sixty milligrams of phosphate caused an inhibition, further slowing the rate of reaction and, after 2 hr., the assay had not yet been completed (Fig. 2). However, these quantities greatly exceed those entrained in the precipitate.

Oxalate recovery was tested in the presence of 2–20 $\mu$M of calcium and magnesium. Amounts of $Pb^{++}$, $Fe^{+++}$, and $Cu^{++}$ of the same order of magnitude as found in the average urinary sample did not interfere with the recovery. The presence of up to 600 mg. of urea and of 250 mg. of dextrose in no way affected the decarboxylation reaction. All recovery experiments were done in a total volume of 3 ml.

The addition of 10 $\mu$M of potassium oxalate to the aliquot of urine prior to precipitation gave recoveries of 85 to 100%. Addition of the

standard to the dissolved precipitate of urinary oxalate in the flask gave similar recoveries. However, these satisfactory recoveries are made possible by inclusion of EDTA in the reaction mixture. When EDTA was omitted, recoveries ranged from 70 to 80%. The addition

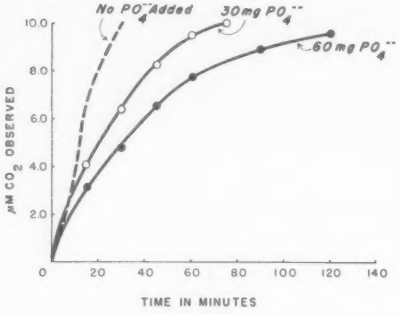


**Fig. 2.** Effect of phosphate on rate of reaction.

of 3 $\mu$M increased the recovery to 85%. A 6-$\mu$M concentration raised the average recovery to at least 95%. EDTA has no effect on the action of the enzyme per se in pure oxalate solutions. It apparently acts on some inhibitor present in urine which is co-precipitated or entrained at *p*H 5.0–5.2. EDTA might prevent interference from anions (chiefly phosphates) (*14*), or it may compete with oxalic acid for a cation such as $Ca^{++}$ or $Mg^{++}$, thus liberating oxalate ion to be acted on by the enzyme.

Twenty-five healthy persons on unrestricted diets contributed 24-hr. specimens for urinary oxalate assay. The average excretion value was found to be 20.5 ± 7.9 (S.D.) mg. $(COOH)_2$/24 hr., with an average concentration of 1.82 mg. $(COOH)_2$/100 ml. In 70 patients suffering from calculus disease, oxalate excretion is not appreciably higher than in normal subjects. It averaged 23.5 ± 9.7 (S.D.) mg./24 hr., with a mean concentration of 1.05 mg. of $(COOH)_2$/100 ml. (Table 1). This supports the findings of Dempsey *et al.* (*15*).

**Table. 1.** Urinary Oxalate Levels per 24 hours in Normal and Calculus Disease Patients

| | *mg. $(COOH)_2$* | | | | | | | |
|---|---|---|---|---|---|---|---|---|
| | *5–10* | *10–15* | *15–20* | *20–25* | *25–30* | *30–35* | *35–40* | *40–45* |
| No. of normals | 1 | 3 | 6 | 10 | 4 | — | — | 1 |
| No. of stone-formers | 1 | 7 | 9 | 23 | 18 | 5 | 4 | 3 |

## Discussion

The enzyme employed in this method is found to be specific, among urinary constituents tested, for oxalic acid. Those methods employing precipitation and subsequent oxidimetric titration are subject to errors from many causes. There is good reason to believe that other organic acids may co-precipitate. The Archer method (*2*) may overestimate the oxalic acid excretion in patients with infections of the urinary tract. The use of extraction of the urine with ether (6–18 hr.) (*4*, *5*) is inconvenient for the handling of a large number of samples. Also, citrates seem to interfere in this procedure (*4*).

The oxalate concentration of average urine samples is insufficient to permit direct assay by the enzymatic technic. When assayed directly, small urine samples show so little $CO_2$ generation that the results are inaccurate. This is overcome by concentrating the oxalate by precipitation with $CaCl_2$ prior to the manometric assay.

The consistent increase in oxalic acid values found in urine after storage might be due to the presence of an urinary enzyme capable of oxidizing glyoxylic acid to oxalic acid. However, the failure of HCl or of freezing to prevent this change speaks against this. Hydrolysis of oxaluric acid is another possible source. Further studies are in process to discover the origin of the extra oxalate.

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# Capillary Blood *p*H by a Dilution Technic

*Arnold G. Ware, Jacques Nowack, and Leola Westover*

**A technic is described for collecting arterialized capillary blood in a small heparinized polyethylene centrifuge tube. The *p*H is measured after dilution and injection into a Beckman micro blood *p*H Assembly. Dilution of the blood with saline results in an average increase of 0.027 *p*H units. This change in *p*H on dilution is eliminated by inclusion of calcium in the diluent.**

**The *p*H of capillary blood drawn and measured as described does not differ significantly from that of undiluted arterial blood drawn from the same patient.**

THE *p*H of whole blood can readily be determined in the clinical laboratory with the use of the Beckman micro blood *p*H assembly. Blood is collected with the necessary precautions in a syringe, the dead-space of which has been filled with sterile heparin. The syringe and contents are transported to the laboratory with a stopper attached to the end of the needle. The blood is injected directly into the electrode assembly. This technic adequately prevents loss of $CO_2$ from the blood to be tested.

The electrode assembly is now available in a unit with thermostatic control. The reference electrode has been elevated to provide improved electrical stability through gravity flow of the conducting fluid. We have used this equipment successfully in our laboratory for several years. Its usefulness and accuracy have been extended by the recent availability of a line-operated, expanded-scale *p*H meter with improved electronic stability.

In order to measure *p*H with this equipment, a blood sample of at least 1 ml. is required. It occurred to us that smaller quantities of

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blood could be used by diluting to this volume. Dryer (*1*) has suggested that dilution with physiological saline does not alter the *p*H of the blood. We, therefore, set out to investigate the practicality of a dilution technic with the hope of using the Beckman electrode assembly for the measurement of capillary blood *p*H.

A procedure has been devised which permits the electrode assembly to be adapted to capillary blood samples. The blood is collected in a heparinized Spinco polyethylene centrifuge tube (volume, 0.4 ml.). The specimen is diluted in a syringe with a special diluent and then injected into the electrode assembly. If the blood is drawn from a prewarmed extremity, the *p*H of the diluted sample does not differ significantly from that of undiluted arterial blood drawn simultaneously from the same patient.

## Methods

### Drawing the Specimen

The extremity (hand or foot) is arterialized by immersion for 5 min. or longer in a pan of water heated to approximately 45°. After drying, a finger or heel puncture is made with a Bard-Parker blade and the blood is allowed to flow directly into a Spinco polyethylene centrifuge tube containing 5 $\mu$l. of U.S.P. heparin (5 U.). The tube is allowed to fill almost to the top, the stopper is inserted and the contents are thoroughly mixed. The specimen should be placed in ice if it is not to be run within 30 to 60 min.

Some practice is required to permit efficient collection of blood in these tubes. The first drop of blood must be forced to the bottom of the tube. This is accomplished by swinging the tube with a "flick of the wrist" so that the blood goes to the bottom. This wets the inside surface of the tube and the remainder of the blood then flows in readily. The tube is filled routinely in less than 30 sec.

### Dilution of the Specimen

A 2-ml. syringe with a 21-gauge needle is filled to the 1.5-ml. mark with diluent. All bubbles are carefully eliminated and the needle is inserted through the cap of the polyethylene tube containing the well-mixed blood specimen. The tube and syringe are then placed in a horizontal position and the tip of the tube is cut off with a sharp blade. The blood is then drawn into the syringe, taking care to position the needle so that no air is introduced. Approximately ¾ of the specimen is drawn into the syringe, the needle is withdrawn, and a stopper is

placed on the end of the needle. The contents are thoroughly mixed before injection into the electrode assembly.

### Injection into the Electrode

Reference buffers or specimens are injected forcibly into the electrode in 0.5- to 1-ml. quantities. The injection pressure must clear the electrode of the preceding solution. The actual volume of fluid held by the electrode is not larger than 0.2 ml. We have found it advisable to rinse between specimens with saline diluent. We also rinse between buffer and specimens. The 2-ml. volume of diluted blood permits duplicate readings.

### *p*H Measurements

All measurements were made at 37°. A Beckman Model 76 expanded-scale *p*H meter was used and the signal was transmitted to a Beckman laboratory potentiometric recorder with a two-fold increase in sensitivity. After injecting the sample into the chamber, the time required for full thermal and *p*H equilibration varied from 30 to 90 sec. Beckman buffers at *p*H 6.86 and 7.40 were used for reference. Buffer settings were made before and after specimen readings to eliminate drifts in excess of 0.002 *p*H units.

## Results

### Precision of Measurements

Replicate measurements were made with a single blood sample injected with and without dilution. Dilutions were made as described with physiological saline which had been neutralized to *p*H 7.0. Extreme care was exercised to mix the blood well before sampling. The reproducibility was comparable with both whole and diluted blood. The standard deviations, calculated from 10 replicate measurements, was 0.006 *p*H units.

### Dilution Error

It was found that dilution of blood with physiological saline caused an increase in the *p*H. The average increase at a 1 to 5 dilution was 0.027 *p*H units. The range of increases varied from 0.010 to 0.040 in specimens from 20 hospitalized patients. The degree of increase in *p*H was not affected appreciably by minor variations in dilution.

### Elimination of Dilution Error

Rodkey (*2*) demonstrated an increase in *p*H which occurs with blood samples on dilution with saline. He provided evidence that this could be prevented by addition of calcium ions and suggested that the calcium and hydrogen ions compete for binding sites on the protein.

We have confirmed Rodkey's findings. The addition of 5 mEq. of calcium per liter of saline diluent appears to eliminate the dilution error. The effect of adding several different concentrations of calcium is demonstrated in Table 1. The data clearly show that calcium influences the *p*H of blood diluted with saline.

Since normal serum contains approximately 5 mEq. of potassium and 2 mEq. of magnesium per liter, we thought it advisable to use a diluent containing these cations as well as calcium. Therefore, a 0.9% sodium chloride diluent was prepared which was supplemented with 5 mEq./L. $CaCl_2$, 5 mEq./L. KCl, and 2 mEq./L. $MgCl_2$. It was shown that there was no significant difference between the *p*H of blood diluted with this diluent and one containing only the calcium (Table 1). Therefore, we have used this diluent containing all three cations purely on the basis that it is closer in composition to normal plasma.

### Comparison of Capillary and Arterial Blood *p*H

Capillary blood *p*H was measured after dilution and compared with the *p*H of whole arterial (brachial or femoral) blood drawn simultaneously from the same patient. The results on 10 randomly selected patients were compared when physiological saline was used to dilute the capillary blood (Table 2). The average increase of capillary over arterial blood *p*H was 0.035. Interestingly enough, this difference was

**Table 1.** Effect of Calcium on the pH of Blood Diluted with Saline

| *Calcium in diluent (mEq./L.)* | *pH Difference (diluted minus whole blood)* | |
|---|---|---|
| | *Average** | *Range* |
| 0 | +0.027 (20) | +0.010 to +0.040 |
| 2.5 | +0.018 (3) | +0.008 to +0.025 |
| 5 | +0.005 (2) | +0.000 to +0.010 |
| 10 | −0.009 (3) | −0.031 to +0.007 |
| 5† | +0.004 (7) | −0.013 to +0.010 |

*Figures in parentheses represent the number of specimens tested.
†Also containing 5 mEq. KCl and 2 mEq. $MgCl_2$ per liter.

**Table 2.** Comparison of pH of Whole Arterial Blood and Capillary Blood Diluted with Saline

| *Patient* | *pH* | | |
|---|---|---|---|
| | *Diluted capillary blood* | *Arterial blood* | *Difference in pH (diluted minus arterial)* |
| 1 | 7.487 | 7.444 | +0.043 |
| 2 | 7.403 | 7.359 | +0.044 |
| 3 | 7.488 | 7.468 | +0.020 |
| 4 | 7.428 | 7.411 | +0.017 |
| 5 | 7.380 | 7.375 | +0.005 |
| 6 | 7.450 | 7.400 | +0.050 |
| 7 | 7.432 | 7.374 | +0.058 |
| 8 | 7.500 | 7.446 | +0.054 |
| 9 | 7.454 | 7.445 | +0.009 |
| 10 | 7.449 | 7.404 | +0.045 |
| | | | +0.035* |

*Average difference.

**Table 3.** Comparison of pH of Whole Arterial Blood and Diluted Capillary Blood

| *Patient* | *Capillary blood pH** | *Arterial blood pH* | *Difference in pH (capillary minus arterial)* |
|---|---|---|---|
| 1 | 7.440 | 7.435 | +0.005 |
| 2 | 7.446 | 7.456 | −0.010 |
| 3 | 7.403 | 7.392 | +0.011 |
| 4 | 7.395 | 7.384 | +0.011 |
| 5 | 7.338 | 7.335 | +0.003 |
| 6 | 7.402 | 7.418 | −0.016 |
| 7 | 7.426 | 7.414 | +0.012 |
| 8 | 7.399 | 7.392 | +0.007 |
| | | | +0.003† |

*Obtained after dilution with saline containing calcium, potassium, and magnesium as described in the text.

†Average difference.

comparable to that observed from the dilution effect of the saline alone (Table 1).

This experiment was repeated on 8 additional patients, in this case the capillary blood being diluted with the saline diluent containing calcium, magnesium, and potassium. The results are shown in Table 3. There was not a significant difference between the *p*H of capillary and arterial bloods. Statistical evaluation of the data provides 95% confidence that paired samples of capillary and arterial blood will differ by no more than 0.027 *p*H units.

## Discussion

The data presented provide evidence that dilution of whole blood with saline results in a slight elevation of *p*H. The change in *p*H which was observed could be eliminated by the addition of calcium to the saline. This confirms the suggestion of others (*2*) that calcium and hydrogen ions compete for binding sites on the protein.

Magnesium may also have a similar effect. Therefore, it was felt desirable to include this ion in the diluent. Potassium was included on the hypothetical basis that this might decrease cation exchanges with the red cell. No evidence was provided that either potassium or magnesium have an effect on the *p*H. However, we prefer to include them in the diluent.

The experiments confirm Gambino's (*3*) claim that the *p*H of arterialized capillary blood agrees closely with that of arterial blood. Gambino's comparisons agreed within somewhat closer limits than ours. This is probably the result of errors produced by our blood collection technic and/or the effects of dilution. However, the differences which we observed are well within acceptable limits.

Brief exposure of the capillary blood to air apparently does not affect the *p*H significantly. We have been successful in collecting the bloods in less than 30 sec. It was demonstrated that slower drawing periods result in significant elevations in *p*H. We have succeeded, however, in drawing two successive tubes of blood with good agreement in *p*H as long as the drawing time of each is less than 30 sec.

It is important to warm the extremity well in order to arterialize the capillary blood. Insufficient warming will result in a lower *p*H, approaching that of the venous blood. It is of interest that the largest difference which we observed between arterial and capillary blood occurred in the blood drawn from a patient in coma. The difference observed (Table 3, Patient 6) was 0.016 *p*H units. This was probably the result of the extremely slow circulation which required a longer time to arterialize the capillary blood.

It is important to suspend the cells thoroughly in the plasma or diluent before sampling or injection into the electrode. This will prevent *p*H changes which may occur between cells and plasma at different temperatures (*4*).

It was demonstrated that the concentration of heparin added to the sample could be varied by several times without changing the *p*H. Therefore, the quantity of heparin is critical only in preventing clot-

ting. We routinely draw two tubes of blood to permit additional measurements in the event of clot formation or if they are desired for other reasons.

It is obviously important to minimize back pressure on the syringe plunger when making dilutions of the blood to prevent changes in *p*H due to loss of $CO_2$.

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# The Fate of Large Doses of Creatine Injected Intraperitoneally into Normal Rats

*John F. Van Pilsum and Richard M. Warhol*

**Four young adult male rats were given an intraperitoneal injection of 227 mg. creatine and their urines collected for 48 hr. The creatine and creatinine in the carcass and urine of these 4 rats and in those of 4 control rats were determined.**

**Eighty-nine per cent of the injected dose was recovered—83% in the urine and 6% in the carcass. The data are interpreted to indicate that there was no significant destruction of creatine under these experimental conditions in the normal rat.**

RECENTLY much has been written about the alteration of kidney transamidinase activities in vitro by various experimental procedures (*1–7*). Walker (*5, 8*) has identified and discussed some of these alterations of kidney transamidinase activities, as alterations in the rate of biosynthesis of creatine.

It is well established that the urinary excretion of creatinine is remarkably constant for man and animals. In fact, it appears to be constant even in animals in which the rates of guanidinoacetic acid synthesis in vitro have been altered greatly. For example, kidneys from both protein-depleted rats and vitamin E–deficient animals had, in vitro, only a fraction of the transamidinase activities of kidneys from normal animals (*1, 2, 6, 9*). Yet the carcasses of the protein-depleted rats contained normal amounts of creatine and creatine phosphate (*2*) and the vitamin E–deficient rabbits had normal amounts of carcass plus urine creatine and creatinine (*6*). If the rates of creatine synthesis in vivo were directly related to transamidinase activities in vitro the constant rates of excretion of creatine plus creatinine would have

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been possible *only* if there were a direct relationship between creatine synthesis in vivo and creatine destruction in vivo.

Some controversy exists about the destruction of creatine in vivo. In 1941, Bloch *et al.* (*10*) fed small amounts of $N^{15}$-labeled creatine (53 mg./kg. body weight per day) to normal rats. They concluded that "The close agreement between the values for daily excretion and synthesis excludes the occurrence of any major metabolic pathway other than that of creatinine formation and excretion." They also stated, "Our findings, obtained with normal animals on a creatine-free stock diet, do not exclude the possibility that even extensive creatine degradation may occur when the compound is administered in large quantities." Later in 1946, Peters and Van Slyke stated in their book, *Quantitative Clinical Chemistry* (*11*), "It is, therefore, hard to conceive that creatinine excretion could remain so constant if all of the variable amounts of creatine produced in metabolism have no choice but to become creatinine. It seems more likely that the amount of creatine converted to creatinine is relatively constant and that creatine in excess of this is disposed of in some other manner."

Amounts of creatine approximately 30 fold greater than those used by Bloch *et al.* (*10*) were injected intraperitoneally into young adult normal rats. The urines were collected for 48 hr. after the injection and the animals were sacrificed. The creatine and creatinine in the carcass and urine were measured. No destruction of the injected creatine could be detected, with practically all of the injected dose excreted in the urine as creatine.

## Methods

Eight male albino Holtzman rats weighing 140–150 gm. were used in the experiment. Eight milliliters of a water solution of creatine at 40° (containing 227 mg. creatine, which is equivalent to 192 mg. of creatinine) was injected intraperitoneally into 4 of the rats. All rats were housed individually in metabolism cages and had access to water and a creatine-free diet (*12*) ad libitum. A glass-wool barrier was placed in the neck of the funnel to prevent the feces from entering the urine. The urines were collected under toluene for 48 hr. after the injection. The rats were killed by a blow on the head, their skins were removed, and the carcasses were weighed. The carcasses were ground in a Toledo Chopper, Model No. 5120-0-009, and the ground carcasses were blended in a Waring Blendor with water and the homogenates diluted to 1 L.

One:twenty Folin-Wu protein-free filtrates were prepared and analyzed for creatine (*13*).

The urine-collection funnels and cages were washed with distilled water and this wash added to the urines. The volumes of the diluted urine were made up to 275 ml. One:ten Folin-Wu filtrates were made and analyzed for creatine and creatinine (*13*).

## Results and Discussion

The values for urine and carcass creatine and creatinine are shown in Table 1. A total of 89% of the injected dose was recovered, which was considered to indicate that there had not been a significant destruction of the injected creatine. Eighty-three per cent of this injected dose was found as urine creatine, 6% as carcass creatine plus creatinine; 1.5% of the injected dose was measured as urinary creatinine.

These data agree, then, with those reported by Bloch *et al.* (*10*) in that within the sensitivity of the methods used, no destruction of creatine could be detected in the normal rats. It is then believed the constancy of carcass and urine creatine plus creatinine in normal and experimental rats cannot be accounted for by an alteration in the destruction of these compounds. It seems more probable that there is a

**Table 1.** FATE OF CREATINE* INJECTED INTRAPERITONEALLY INTO NORMAL RATS

| *Group* | *Rat No.* | *Carcass + urine creatinine + creatine* | *Carcass creatinine + creatine* | *Urine creatinine + creatine* | *Urine creatinine* | *Urine creatine* |
|---|---|---|---|---|---|---|
| 1. Control | | | | | | |
| | 1 | 221 | 212 | 9 | 7 | 2 |
| | 2 | 207 | 196 | 11 | 7 | 4 |
| | 3 | 218 | 212 | 6 | 4 | 2 |
| | 4 | 189 | 181 | 8 | 6 | 2 |
| AVERAGE | | 209 | 200 | 8 | 6 | 2 |
| 2. Creatine injected | | | | | | |
| | 1 | 383 | 222 | 161 | 8 | 153 |
| | 2 | 378 | 214 | 164 | 10 | 154 |
| | 3 | 367 | 200 | 167 | 9 | 158 |
| | 4 | 393 | 212 | 181 | 8 | 173 |
| AVERAGE | | 380 | 212 | 168 | 9 | 160 |
| Differences of averages of Groups 1 & 2 | | 171 | 12 | 8 | 3 | 150 |
| % Recovery | | 89 | 6 | 83 | 1.5 | 82 |

*All values were expressed as mg. creatinine.

relatively constant synthesis of creatine in vivo regardless of the rate of synthesis of guanidinoacetic acid in vitro in kidney homogenates.

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# Plasma and Erythrocyte Enzymes in Phenylhydrazine Anemia

*Max M. Friedman, Bernard Lapan, and Bernard S. Kahn*

**Dehydrogenases and transaminases occur in high activities in the erythrocyte when compared to the plasma in which the erythrocyte is suspended. Destruction of the erythrocyte with phenylhydrazine does not release any enzyme activity into the plasma.**

SEVERAL YEARS AGO LaDue (*2*) made the interesting observation that soon after myocardial infarction the plasma glutamic oxalacetic transaminase is increased, reaches a peak within a few days, and then returns to normal. This observation stimulated a new area of clinical interest in diseases related to infarction and necrosis as manifested in plasma enzymes changes.

The source of this increased plasma enzyme activity in disease has become of interest to the clinical chemist. Is this activity newly formed, or is the enzyme system transported from the necrotic tissue to the circulating plasma to be excreted or inactivated? Experimental work has made it obvious that these enzymes are not excreted into the urine. Where or how they are inactivated would be more difficult to demonstrate. Transport from necrotic tissue to plasma assumes that the "leaching" process transfers the complete kinetic system from the fixed tissue phase to the circulating plasma phase. The complexity of the problem is of course inherent in the complexity of protein structure and synthesis and the kinetics of the enzyme systems.

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A preliminary report was presented at the meeting of the American Association of Clinical Chemists, Aug. 31, 1961, New York City (*1*).

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There is no evidence for the identity of tissue and plasma enzyme systems, although its establishment has now been attempted immunochemically (*3*). However, the assumption has been made generally that the increased plasma enzyme activity in necrotic disease has its origin in the affected tissue (*4*). Circumstantial evidence points to the tenability of this assumption since plasma acid phosphatase is increased in prostatic disease, amylase in pancreatic disease, and transaminase in liver and coronary disease. But this concept needs be accepted with reservations until definitive evidence is forthcoming that the enzyme system is transported from necrotic cell to circulating plasma.

It occurred to us that, since the erythrocyte contains large amounts of enzyme activity and is suspended in the circulating plasma, which itself has comparatively negligible activity, then the destruction of the erythrocyte should result in increased plasma activity if this were merely a process of enzyme transfer. For this experiment rabbits were injected daily with phenylhydrazine to obtain erythrocyte destruction and the enzyme activities followed in the plasma at various times after the injection. Lactic, malic, glucose-6-phosphate dehydrogenases, and glutamic oxalacetic transaminase were studied. The data show that despite extensive erythrocyte destruction and erythrocyte enzyme loss there was no apparent change in plasma enzymes.

## Materials and Methods

The injection of phenylhydrazine HCl in rabbits (25 mg. per injection) and the preparation of washed erythrocytes were carried out as previously described (*5*). The plasma was obtained at the first centrifugation and hemolyzed plasma discarded. Microhematocrits were performed on an aliquot of the same blood specimens.

Enzyme assays for the dehydrogenases were performed as previously described (*5*). Glutamic oxalacetic transaminase was determined spectrophotometrically by the method of Karmen (*6*). The enzyme units were calculated as the millimicromoles of TPN reduced, or DPNH oxidized, per milliliter of whole blood or plasma per minute at 25°. Calculations were based on the molar extinction coefficient of pyridine nucleotides as determined by Horecker and Kornberg (*7*).

## Results and Discussion

The data for enzyme activities are shown in Tables 1–4. The "zero" day represents blood drawn immediately before injection, and the

**Table 1.** LACTIC DEHYDROGENASE* IN WHOLE BLOOD AND PLASMA OF RABBITS RECEIVING DAILY INJECTIONS OF PHENYLHYDRAZINE

| *Rabbit No.* | *Injection days* | *Hemoglobin %* | *Whole blood† (U./ml.)* | *Plasma (U./ml.)* |
|---|---|---|---|---|
| 1 | 0 | 11.4 | 8700 | 16 |
| | 4 | 3.5 | **6050** | 24 |
| 2 | 0 | 10.6 | 8350 | 21 |
| | 4 | 4.3 | **4950** | 27 |
| | 6 | 3.1 | **5900** | 13 |
| 3 | 0 | 13.9 | 7350 | 26 |
| | 3 | 6.0 | **4150** | 20 |
| 4 | 0 | 11.1 | 7100 | 19 |
| | 3 | 4.7 | **4100** | 11 |

*Enzyme units in millimicromoles DPNH per milliliter whole blood or plasma per minute at 25°.

†Boldface figures are units of activity "dissipated" from whole blood as result of red blood cell destruction.

subsequent days are shown in the tables. The unit activities for whole blood on zero day, shown in the fourth columns, were determined on washed erythrocytes and then calculated for whole blood on the basis of hematocrit. The boldface figures for activities "dissipated" were calculated from the erythrocytes destroyed. Using the data for Rabbit 1 in Table 1 a sample calculation for lactic dehydrogenase would be as follows. Before injection the hemoglobin was 11.4% and the whole blood had an LDH activity of 8700 U./ml. After the fourth daily injec-

**Table 2.** MALIC DEHYDROGENASE* IN WHOLE BLOOD AND PLASMA OF RABBITS RECEIVING DAILY INJECTIONS OF PHENYLHYDRAZINE

| *Rabbit No.* | *Injection days* | *Hemoglobin %* | *Whole blood† (U./ml.)* | *Plasma (U./ml.)* |
|---|---|---|---|---|
| 1 | 0 | 10.6 | 4350 | 13 |
| | 4 | 3.0 | **2870** | 12 |
| 2 | 0 | 13.0 | 4700 | 13 |
| | 4 | 4.5 | **3080** | 10 |
| 3 | 0 | 10.2 | 4380 | 18 |
| | 4 | 2.9 | **3120** | 18 |
| 4 | 0 | 10.4 | 3770 | 11 |
| | 6 | 4.1 | **2210** | 23 |
| 5 | 0 | 8.9 | 4130 | 10 |
| | 6 | 3.7 | **2410** | 26 |

*Enzyme units in millimicromoles DPNH per milliliter whole blood or plasma per minute at 25°.

†Boldface figures are units of activity "dissipated" from whole blood as result of red blood cell destruction.

tion the hemoglobin was reduced to 3.5% and represents a loss of 7.9 gm. hemoglobin per 100 ml. The enzyme activity lost in the destruction of this amount of hemoglobin is the 6050 U./ml. in boldface figures. All other boldface figures are based on the same type of calculation. Actually these are minimum values since the hemoglobin concentration *after phenylhydrazine injections also represents that for newly* formed reticulocytes, so that the actual loss of enzyme activity is even larger than shown.

Table 1 shows conclusively that red blood cell destruction does not result in any increase in plasma activity. The slight variations seen in the plasma column are within the experimental error. This compares with a thirty-fold increase in plasma LDH of rats who received carbon tetrachloride intraperitoneally (*8*). Thus it appears that despite the relatively large enzyme activities that disappear as a result of erythrocyte destruction there is no significant gain in plasma activities.

Malic dehydrogense (Table 2) and glucose-6-phosphate dehydrogenase (Table 3) show the same results as for lactic dehydrogenase. Here, also, large amounts of enzyme activities are "dissipated" as a result of red blood cell destruction, while plasma changes are negligible and within experimental error.

Glutamic oxalacetic transaminase (Table 4) is of clinical interest because of its extensive use in the diagnosis of diseases in which tissue necrosis occurs. And here, also, no significant changes in this enzyme activity can be seen in the plasma after erythrocyte destruction.

This experiment gives no indication as to the disposal of the enzyme

**Table 3.** GLUCOSE-6-PHOSPHATE DEHYDROGENASE* IN WHOLE BLOOD AND PLASMA OF RABBITS RECEIVING DAILY INJECTIONS OF PHENYLHYDRAZINE

| *Rabbit No.* | *Injection days* | *Hemoglobin %* | *Whole blood†* (*U./ml.*) | *Plasma* (*U./ml.*) |
|---|---|---|---|---|
| 1 | 0 | 14.6 | 844 | 0.9 |
| | 3 | 5.6 | **520** | 1.1 |
| 2 | 0 | 14.6 | 1030 | 1.0 |
| | 3 | 6.1 | **600** | 1.4 |
| 3 | 0 | 13.9 | 820 | 0.7 |
| | 3 | 6.0 | **465** | 0.8 |
| 4 | 0 | 11.1 | 1000 | 0.7 |
| | 3 | 4.7 | **575** | 1.2 |

*Enzyme units in millimicromoles TPN per milliliter whole blood or plasma per minute at 25°.

†Boldface figures are units of activity "dissipated" from whole blood as result of red blood cell destruction.

Table 4. Glutamic Oxalacetic Transaminase* in Whole Blood and Plasma of Rabbits Receiving Daily Injections of Phenylhydrazine

| *Rabbit No.* | *Injection days* | *Hemoglobin %* | *Whole blood†* (U./ml.) | *Plasma* (U./ml.) |
|---|---|---|---|---|
| 1 | 0 | 10.8 | 295 | 12 |
| | 3 | 6.2 | **126** | *10* |
| 2 | 0 | 13.0 | 270 | 16 |
| | 4 | 4.5 | **177** | 24 |
| 3 | 0 | 8.9 | 180 | 6 |
| | 6 | 3.7 | **105** | 13 |
| 4 | 0 | 10.2 | 245 | 25 |
| | 6 | 2.9 | **175** | 10 |

*Enzyme units in millimicromoles DPNH per milliliter whole blood or plasma per minute at 25°.

†Boldface figures are units of activity "dissipated" from whole blood as result of red blood cell destruction.

activities from the red blood cells after their destruction, but they apparently do not appear in the plasma and any search for it in other body fluids or tissues would be futile. Since the red blood cell is suspended in the plasma this experiment suggests that the disposition of enzymes from necrotic cells is not one of mere transport to the plasma. The destruction of the red blood cell and the loss of its enzyme content is analogous to the necrosis of tissue cells, and yet with extensive erythrocyte destruction there is no increase of plasma enzyme activity. It should be recognized that with enzymes one deals not only with a chemical entity (apoenzyme) which is itself a complex substance, but rather with a complicated kinetic system. To think in terms of simple transfer of this kinetic system from one biologic phase to another is to underestimate the problem.

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# The Stability of Glucose in Serum

*Joseph Ruiter, Frederick Weinberg, and Andrew Morrison*

**The destruction of glucose does not occur in separated serum during the 48 hr. immediately following collection.**

**If necessary, the determination of the glucose level of serum can be delayed at least 48 hr. without introducing a significant error, provided the serum is removed from the clot immediately after collection.**

It has long been known that in the presence of formed elements of the blood, glucose is broken down in vitro (*3, 6, 7*). It seems to have been generally assumed that enzymes in the plasma were also glycolytic and that prolonged standing of plasma or serum even in the absence of formed blood elements would result in low glucose levels (*1, 2, 5*). The introduction of automatic analysis,* which uses dialyzed serum, requires that this idea be tested. Further, it was thought desirable to do this in order to determine whether the addition of antiglycolytic agents, such as fluoride or thymol, would be necessary.

This study sought to establish the stability of glucose in serum. The glucose concentration of serum permitted to remain in contact with the clot was compared to the glucose concentration of the same serum removed from the clot shortly after collection. Determinations were made at 24-hr. intervals for 3 consecutive days, during which time the specimens remained at room temperature and no attempt was made to preserve the sugar.

## Method

1. From 25 patients selected at random, 15 ml. of blood was collected into clean, dry tubes by venipuncture and allowed to stand at room temperature to clot.

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*AutoAnalyzer, Technicon Instruments Co., Chauncey, N. Y.

2. The blood stood for ½–2 hr. before being centrifuged.

3. From each tube 2 ml. of the clear serum was removed and placed in separate 2-ml. polystyrene cups. The remainder of the serum was kept in the tube in contact with the clot.

4. The glucose level was determined by a modification of the Hoffman procedure utilizing the potassium ferricyanide–potassium ferrocyanide oxidation-reduction reaction as recommended for use with the automatic system (*4, 8*).

5. After each run the specimens were set aside at room temperature for 24 hr., after which time the glucose level of each sample was again determined.

6. Meanwhile, the original tubes containing the clot were centrifuged, sufficient serum decanted, and the glucose level of this (clot exposed) serum measured. The excess serum not required for determina-

**Table 1.** GLUCOSE LEVELS IN SERUM REMOVED FROM CLOT IMMEDIATELY AFTER COLLECTION

| | *(Glucose level (mg./100 ml.)* | | |
|---|---|---|---|
| *Specimen No.* | *Immediately after collection* | *After 24 hr. at room temperature* | *After 48 hr. at room temperature* |
| 1 | 100 | 100 | 103 |
| 2 | 77 | 76 | 76 |
| 3 | 104 | 106 | 109 |
| 4 | 135 | 138 | 137 |
| 5 | 119 | 118 | 118 |
| 6 | 66 | 68 | 68 |
| 7 | 85 | 86 | 86 |
| 8 | 60 | 60 | 54 |
| 9 | 164 | 164 | 162 |
| 10 | 72 | 73 | 75 |
| 11 | 171 | 165 | 152 |
| 12 | 148 | 149 | 138 |
| 13 | 140 | 138 | 138 |
| 14 | 84 | 83 | 80 |
| 15 | 70 | 69 | 66 |
| 16 | 70 | 65 | 64 |
| 17 | 70 | 66 | 66 |
| 18 | 63 | 62 | 64 |
| 19 | 48 | 49 | 53 |
| 20 | 76 | 74 | 74 |
| 21 | 156 | 158 | 156 |
| 22 | 169 | 174 | 172 |
| 23 | 97 | 96 | 97 |
| 24 | 95 | 93 | 95 |
| 25 | 128 | 131 | 130 |

tion of glucose at the first and twenty-fourth-hour determinate was allowed to remain in contact with the clot.

7. Upon completion of this series the tubes and cups were set aside until the following day.

8. Steps 5 and 6 were repeated on the third day, 48 hr. after the blood was drawn.

## Results and Discussion

Table 1 illustrates the level of reducing substances in the serums which had been removed from the clot immediately after collection. It is apparent that the level remains constant for at least 48 hr. at room temperature.

Table 2 however, shows the marked changes that occur in the glucose level when the serum remains in contact with the clot. Glycolysis is profound and prompt.

**Table 2.** Glucose Level in Serum Remaining in Contact with Clot

| | (*Glucose level (mg./100 ml.)* | | |
|---|---|---|---|
| *Specimen No.* | *Immediately after collection* | *After 24 hr. at room temperature* | *After 40 hr. at room temperature* |
| 1 | 100 | 49 | <10 |
| 2 | 77 | 12 | <10 |
| 3 | 104 | 24 | <10 |
| 4 | 135 | 70 | <10 |
| 5 | 119 | 31 | <10 |
| 6 | 66 | <10 | <10 |
| 7 | 85 | 24 | <10 |
| 8 | 60 | 18 | <10 |
| 9 | 164 | 92 | 15 |
| 10 | 72 | 16 | <10 |
| 11 | 171 | 86 | <10 |
| 12 | 148 | 42 | <10 |
| 13 | 140 | 67 | <10 |
| 14 | 84 | 20 | <10 |
| 15 | 70 | 34 | <10 |
| 16 | 70 | 20 | <10 |
| 17 | 70 | 28 | <10 |
| 18 | 63 | 27 | <10 |
| 19 | 48 | 23 | <10 |
| 20 | 76 | 30 | <10 |
| 21 | 156 | 96 | 16 |
| 22 | 169 | 122 | 55 |
| 23 | 97 | 20 | 20 |
| 24 | 95 | 26 | 19 |
| 25 | 128 | 86 | 24 |

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# Direct Determination of Calcium in Icteric Serum

*B. Fingerhut and H. Miller*

**A rapid method suitable for the direct titrimetric determination of calcium in icteric sera is presented. The major portion of serum chromogens are oxidized to colorless products by either a mixture of hydrogen peroxide and potassium ferricyanide or by sodium hypochlorite. Ethylenediamine tetraacetic acid (EDTA) is the titrant used and either calcein or Cal-Red is suitable as the specific calcium indicator. Results compare favorably with those obtained by the method of Clark and Collip.**

THE SIMPLICITY with which the direct titrimetric determination of serum calcium can be performed with specific calcium indicators and ethylenediamine tetraacetic acid (EDTA) as chelating agent has contributed to its increased use in clinical chemistry laboratories. One difficulty encountered with the use of these calcium indicators is poor end-point detection in highly pigmented specimens (*1, 2*). Spectrophotometric determination of the end-point has been used to overcome this difficulty (*3, 4*). Nevertheless these methods are more cumbersome and tedious than titrimetric procedures.

Two simple methods for removing the interfering effect of serum pigments are presented in this communication. The methods are based upon the ability of a mixture of hydrogen peroxide and potassium ferricyanide, or of sodium hypochlorite alone, to oxidize quickly the major portion of serum chromogens to colorless products at room temperature. The resultant decolorized mixture can then be adjusted to the high *p*H required and titrated with EDTA with little color interference, using either calcein* (*5*) or Cal-Red† (*6*) as the calcium indicator.

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*Calcein-Fisher Scientific Products, New York, N. Y.
†Cal-Red Scientific Service Laboratories, Dallas, Tex.
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## Method

Into a small test tube, (4 × ½ in.) place 0.1 ml. serum or standard and 0.2 ml. 3% (w/v) $H_2O_2$, followed by 0.1 ml. of 0.25% (w/v) $K_3Fe(CN)_6$ if calcein is to be used as the indicator, or 0.2 ml. of 0.5% (w/v) NaOCl if Cal-Red is the desired indicator. Mix and allow to stand 3 min. or longer. Add one drop 10% (w/v) KCN; mix. Just before titrating with 0.05% (w/v) EDTA (disodium salt), add 0.2 ml. 10% (w/v) KOH, one drop n-octyl alcohol, and approximately 10 mg. calcein-KCl-thymolphthalein dye mixture* or 0.1 ml. 1% (w/v) Cal-Red solution.

The titration is performed with a Gilmont ultramicroburet of 1.0-ml. capacity. Mixing during the titration is accomplished by a fine stream of compressed air entering beneath the surface of the solution. The color change at the end point is from green to purple with calcein, and pink or light brown to a pale green or blue color with Cal-Red. The color changes vary somewhat with the concentration of residual serum pigments.

## Results

The procedure using calcein dye as the indicator yields consistently lower results than those obtained by the Clark-Collip (*7*) procedure. A preliminary series of 60 specimens analyzed by both methods averaged 0.25 mg./100 ml. lower for the calcein procedure. The results of a series of 17 specimens having abnormal bilirubin concentrations similarly indicate lower results for the calcein procedure. The average difference for this series is 0.25 mg./100 ml. The standard deviation between results using the Clark-Collip and those obtained using calcein procedures is 0.38 mg./100 ml.

Results obtained using the Cal-Red method agree more closely with those of the Clark-Collip procedure than do those obtained using the calcein procedure. In a series of 30 analyses, the dye procedure values averaged 0.09 mg./100 ml. lower than Clark-Collip values. Values with the series of 17 specimens (Table 1) also averaged 0.09 mg./100 ml. less than the Clark-Collip results. The standard deviation of results between these two procedures is 0.17 mg./100 ml.

*This reagent consists of a finely ground mixture of 200 mg. calcein, 120 mg. thymolphthalein, and 20 gm. of potassium chloride. Approximately 10 mg. of this mixture is dispensed by using the small amount of dye which can be held in the bevel of a 20-gauge hypodermic syringe needle.

**Table 1.** Comparison of Serum Calcium Results by the Clark-Collip, Calcein, and Cal-Red Methods

| *Specimen No.* | *Bilirubin conc.* | *Serum calcium (mg./100 ml.)* | | | | |
|---|---|---|---|---|---|---|
| | | *Clark-Collip* | *Calcein* | *Difference between Clark-Collip and Calcein* | *Cal-Red* | *Difference between Clark-Collip and Cal-Red* |
| 1 | 6.1 | 10.0 | 9.7 | +0.3 | 10.0 | 0.0 |
| 2 | 5.0 | 10.0 | 9.4 | +0.6 | 10.0 | +0.0 |
| 3 | 8.2 | 9.4 | 9.1 | +0.3 | 9.3 | +0.1 |
| 4 | 13.8 | 10.2 | 9.5 | +0.7 | 10.0 | +0.2 |
| 5 | 5.0 | 9.2 | 9.0 | +0.2 | 9.3 | −0.1 |
| 6 | 3.4 | 8.8 | 9.0 | −0.2 | 9.0 | −0.2 |
| 7 | 0.8 | 9.7 | 9.4 | +0.3 | 9.5 | +0.2 |
| 8 | 1.1 | 8.8 | 8.5 | +0.3 | 8.6 | +0.2 |
| 9 | 0.8 | 9.1 | 8.6 | +0.5 | 9.0 | +0.1 |
| 10 | 0.5 | 9.2 | 8.6 | +0.6 | 9.0 | +0.2 |
| 11 | 0.3 | 9.2 | 9.2 | 0.0 | 9.1 | +0.1 |
| 12 | 0.5 | 8.9 | 9.0 | −0.1 | 9.0 | −0.1 |
| 13 | 17.0 | 9.0 | 8.7 | +0.3 | 8.7 | +0.3 |
| 14 | 10.4 | 10.1 | 10.0 | +0.1 | 10.0 | +0.1 |
| 15 | 9.2 | 10.3 | 10.2 | +0.1 | 10.1 | +0.2 |
| 16 | 11.8 | 9.4 | 9.0 | +0.4 | 9.2 | +0.2 |
| 17 | 11.2 | 8.8 | 8.5 | +0.3 | 8.8 | 0.0 |
| | | Ave.: 9.42 | Ave.: 9.14 | S.D.: 0.377 | Ave.: 9.33 | S.D.: 0.173 |

The color change exhibited at the end-point with the indicator calcein is nearly completely obscured in specimens from highly jaundiced subjects. Cal-Red, on the other hand, undergoes a color change at the end-point which is visible in the presence of appreciable amounts of serum chromogens. However, the color at the end-point with Cal-Red is a dark green instead of the usual blue or pale green obtained with standards or normally pigmented sera. The use of bile pigment oxidants enables detection of the end-point with calcein and also allows the formation of the more usual blue or pale green end-point with Cal-Red.

The titration volume should be kept at a minimum to facilitate detection of the end-point. Several determinations were carried out using a buret of 2.0-ml. capacity. However, these results were not as reproducible as those obtained with burets of 1.0-ml. capacity or less.

Serum pigments are slowly oxidized at room temperature by either dilute hydrogen peroxide (*8*) or by potassium ferricyanide. However, the sequential addition of hydrogen peroxide and potassium ferricyanide affords more effective and rapid serum decolorization. The

serum-peroxide-ferricyanide mixture remains yellow until the potassium hydroxide is added. Although alkali addition increases the rate of bilirubin oxidation, the hydroxide should be added just before titration. Erratic results are obtained with serum specimens which have been allowed to remain in contact with alkali for several minutes (*6*). The length of time the oxidizing agents remain in contact with serum does not appear to affect the results. Under the conditions outlined in the procedure, serum pigment decolorization is about 70–75% complete when peroxide-ferricyanide is used, and about 65–70% complete when hypochlorite is the oxidizing agent. This was determined by spectrophotometric comparison of the absorbance difference at 420 mμ of a diluted alkaline serum specimen with that of the same specimen similarly diluted after treatment with either peroxide-ferricyanide or hypochlorite.

Although the results reported in Table 1 were obtained using peroxide-ferricyanide with calcein as the indicator and sodium hypochlorite with Cal-Red as the indicator, it is possible to use either oxidizing system with either dye by slightly modifying the procedure. Cal-Red is not very stable in the presence of a peroxide-ferricyanide mixture; therefore, an additional 0.1 ml. of Cal-Red should be used with this combination of oxidizing agents and dye. If sodium hypochlorite is used in conjunction with the calcein dye mixture, it will be noted that the end-point change is from green to pink instead of green to purple because the thymolphthalein portion of the indicator mixture is oxidized.

Results obtained with EDTA procedures presented above are slightly lower than those obtained by the Clark-Collip method. This is in agreement with other investigations reported in the literature (*2, 6, 9, 10*). In addition, some recent listings (*11, 12*) of normal ranges for serum calcium present two sets of values; one for EDTA methods, which range from 8 to 11.5 mg./100 ml. Ca, and another for unspecified procedures similar to the Clark-Collip normal range (9–11.5 mg./100 ml. Ca).

Several other specific calcium indicators (*2*) are available for use with EDTA. These indicators were not tested for their suitability with the oxidizing agents used in the procedures presented. However, if they are stable to oxidation by either peroxide-ferricyanide mixture or hypochlorite, they can probably be substituted for calcein or Cal-Red in the methods outlined above.

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# SCIENTIFIC NOTE

## The Prevention of Cross-Contamination in PBI Determinations

*Wilma L. White*

THE USE of heavy aluminum foil in our ashing procedure for PBI (*1*), introduced 3 years ago, has proved extremely successful in preventing cross-contamination. A Temco double-door muffle furnace is used for which three steel buckets were made; small buckets, 4 in. wide, 6 in. long, and 6 in. deep, and one large bucket, 6 in. wide, 8 in. long, and 6 in. deep. A steel shovel was made to aid the technician in introducing and removing the buckets from the furnace.

The tubes are prepared as follows. A piece of aluminum foil, about 10 in. long and 4½ in. wide is cut. A pair of tubes (duplicate specimens) is placed approximately 2½ in. from the top edge of the foil. The sides are folded together and the foil wrapped up from the bottom. The covering stands at least 2½ in. above the top of the tubes. The aluminum foil should extend to at least this height to prevent the vapors from flowing into neighboring tubes and causing contamination of other specimens. The rising vapors in the wrapped tubes are held within the aluminum foil cylinder and directed back down into the tube. The foil-wrapped tubes are stood upright in the bucket and heated for 3 hr. at 600° ± 10°, after which they are removed from the furnace and cooled to room temperature. The used foil is discarded.

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From the Clinical Chemistry Laboratory, Barnes Hospital, St. Louis 10, Mo.
Received for publication January 20, 1962.

# BOOK REVIEWS

**The Parathyroids.** Roy O. Greep and Roy V. Talmage. Charles C Thomas, Publisher, Springfield, Ill. $12.25

The current problems of the parathyroids, hormone isolation and purification, standardization and relation to homeostasis and kidney function are reviewed by a host of experts in the field.

Although this is not a standard textbook, Dr. McLean does discuss the evolution of the parathyroid gland and some of the basic principles of parathyroid function.

The information gleaned in this book is of a type not found in standard texts (e.g., the application of tissue culture, the chemistry and extraction of the polypeptides possessing parathyroid activity, biologic assay, electron microscopy, etc.).

Any laboratory interested in the special problems of the parathyroids should have this panel of experts available at their fingertips.

The sole criticisms are that the interspersed eulogies to pioneers in the field and the question and answer period at the end of each chapter slow the otherwise fluent and logical trend of this excellent symposium.

*New York Medical College*
*New York 29, N. Y.*

Victor Tchertkoff, M.D.

**An Introduction to Blood Group Serology (ed. 2).** Kathleen E. Boorman and Barbara E. Dodd. Little, Brown & Co., Boston, Mass., 1961.

This compact manual was written for the laboratory technician interested in blood grouping and blood banking. It is straightforward in its approach and covers the fundamentals of blood grouping, laboratory maintenance, various aspects of transfusion, erythroblastosis, and the hemolytic anemias. A brief section on leukocyte and platelet antibodies as well as recent advances in the study of blood groups make this book interesting to the more advanced reader as well.

This book should be useful if its limitations are kept in mind.

*Metropolitan Hospital*
*New York 29, N. Y.*

Eric J. Wake, M.D.
*Director of Blood Bank*

# LETTERS TO THE EDITOR

*To the Editor:*

In an editorial published in 1958 (*Am. J. Clin. Path.* **29,** 479), the need for checking calibrations of volumetric pipets was pointed out. Since that time, numerous calibration checks made in our laboratories reveal that, although much improvement has been made by manufacturers of pipets, the vigilance cannot yet be relaxed.

Recently, the calibrations of a group of 14 pipets were checked, and of these only 6 delivered amounts within the tolerances specified by the manufacturer. Indeed, one of these pipets showed an incredible error of +9%. Such instruments are a menace in any laboratory, and the sin is multiplied if a grossly inaccurate pipet is used to measure a standard solution.

During the past few years, pipets from at least three manufacturers have been found to contain errors exceeding the tolerances claimed by the manufacturers. It must be pointed out that these pipets were all from companies which purport to specialize in making pipets. The fact that most of the errors found were tolerable in terms of the analytic methods in which the pipets are used does not absolve the manufacturers from the responsibility of either *meeting the tolerances they claim or claiming the tolerances they meet.*

The writer has made the following observations based on conversations and correspondence with manufacturers and distributors of pipets: (1) Pipet sales are promoted largely on the basis of price, appearance, durability, and "gimmicks" (colors, personalized inscriptions, and the like). (2) In some cases, neither salesmen nor company officials have adequate working knowledge of calibration technics or tolerances. (3) Some companies seem sincere about *wanting* to put out a quality product but do not hesitate to continue to sell their pipets while endeavoring to make their *claimed* tolerances a reality.

The problem seems to be intermittent; hence a certain type of pipet will conform to stated tolerances for several months, then suddenly the calibrations will be inaccurate for some time. This demonstrates a lack of effective quality control on the part of the manufacturer.

When confronted with a complaint about pipet calibrations, some manufacturers reply that since they have a large number of customers and only a few (or one) complaints, their pipets must be acceptable. The fallacy of this reasoning lies in the fact that the practice of checking pipet calibrations is restricted to relatively few laboratories. Consequently, many are living in blissful ignorance and unknowingly encouraging slipshod manufacturing practices.

The fact that some manufacturers have effective quality-control programs is demonstrated by the regularity with which their calibrated apparatus meets stated tolerances. Healthy competition is certainly to be encouraged in this area, but it is clear that only pressure from the users of pipets will impress

some manufacturers with the importance of accuracy and uniformity of calibration.

The purpose of this letter is not to create undue alarm or to destroy well-founded faith, but rather to encourage a critical attitude toward calibrated apparatus. Clinical chemists, in their eagerness to investigate more esoteric methodologies and instrumentation, should not overlook the importance of controlling the quality of their basic volumetric equipment.

*Massachusetts Memorial Hospitals* JOSEPH S. ANNINO
*Boston 18, Mass.*

*Dear Sir:*

The observation of Gambino (*Clin. Chem.* **9**:110, 1963) that the *p*H of buffers and buffer-serum mixtures should be reported at the temperature of the final reaction mixture is an apt one. However, it is not original; indeed it is well known and practiced by most competent chemists.

In the work we reported on lipase assay (*Clin. Chem.* **8**:509, 1962) we measured the *p*H of our buffer at 37°. The *p*H of the mixture after addition of serum (range, 7.82–8.01) was likewise measured at 37°. Perhaps we erred in not stating this; on the other hand it might be reasoned that such a procedure is a standard technique and need not be included in the report.

It is an interesting sidelight that today most clinical chemists are able to measure *p*H at 37° more conveniently than at any other temperature. Gambino himself has been in no small measure responsible for this. Perhaps a mythical Argus will guide our virtue when using the subtleties of *p*H measurement.

*Harper Hospital* R. P. MACDONALD
*Detroit, Mich.*

the Clinical Chemist

# News Section

## Precongress Symposium

A symposium on "Proteins, Genes and Evolution" based on researches conducted in the Toronto area, will be held on Saturday, Aug. 17, 1963, from 9:00 A.M. until noon, at the Toronto General Hospital. The chairman of the symposium will be Dr. Oliver Smithies. The symposium will include major papers on haptoglobins, serum cholinesterases, comparative biochemistry of insulin, and alpha-2-globulin. Tours of the clinical chemistry laboratories of the teaching hospitals in Toronto will be arranged for the afternoon. Those desiring further information are urged to contact DR. C. J. PORTER, Department of Biochemistry, Toronto General Hospital, Toronto 2, Ontario.

## Section News

Section Secretaries are reminded of the following deadlines for submission of information concerning their activities: August issue, June 11; October issue, Aug. 13; and December issue, Oct. 1. Observance of these deadlines will assure prompt publication.

### Chicago Section

The Chicago Section has held four major meetings this season, one of which was devoted to the biochemistry of the fibrinolytic system; the speaker was Dr. Frederick W. Pairent. In November Dr. Rudi Schmid of the University of Chicago spoke on bilirubin metabolism. In January the section held a workshop on analytical methods for blood gases, including demonstrations of technics and discussion. In February Dr. Robert Wissler of the University of Chicago spoke on fatty acid and lipid relationships in primate atherosclerosis.

### Philadelphia Section

In January the section met jointly with the local section of the American Chemical Society. Dr. Morris A. Spirtes spoke on the mode of action of psychotherapeutic drugs. Dr. Spirtes is associated with Hahnemann Medical College, Department of Pharmacology. Later in the month Dr. J. H. Boutwell of Temple University Medical Center led a discussion of the results of an interlaboratory survey on enzyme determinations. The effects of temperature, substrate, and other

factors were reviewed. Applicability of international units was discussed and problems associated with the definites of units were considered. In February the section held a joint meeting with the Pathological Society of Philadelphia. The program was entitled, "The Biochemistry of the Cancer Cell," with the major address by Dr. Sidney Weinhouse of Temple Medical Center. At the monthly ChemiClinic Dr. Boutwell discussed calcium methodology, and Dr. Alan Mather reviewed existing methods for serum iron and iron-binding globulins. Dr. Mather is associated with the Memorial Hospital of Wilmington, Del. In March Dr. Seymour Winsten of the Albert Einstein Medical Center reviewed the assay of ketosteroids and hydroxycorticosteroids. The regular scientific meeting was held jointly with Southeastern Pennsylvania Chapter of the American Society of Microbiologists. Dr. Leonard Korngold of the New York Hospital for Special Surgery discussed a qualitative test for multiple myeloma and macroglobulinemia. He discussed results obtained with immuno-electrophoresis and agar-gel diffusion technics. The April ChemiClinic was moderated by Meyer Samson of Samson Laboratories. Mr. Samson discussed cross-contamination in the PBI determination.

### Capital Section

The first meeting of the season was held at the Armed Forces Institute of Pathology. Following reports of the officers, Dr. Julius Sendroy presented a film on the basic process of automatic analysis in clinical chemistry. Lt. Col. J. N. Stabile discussed practical aspects of automation. The second meeting of the season was held jointly with the local section of the Society for Experimental Biology and Medicine. A series of four papers were presented covering injection of chemicals into chicken eggs in toxicity testing; the determination of serum albumin by dye-binding technics; histochemical aspects of biochemical differentiation; and the retention of sialic acid, protein, and DNA by cultured mammalian cells following freezing injury.

### Puget Sound Section

The monthly meetings of the Puget Sound Section are held at local hospitals and clinics, and are attended by all persons interested in clinical chemistry.

In January, Cecil Hougie, Associate Professor of Pathology, Director of Clinical Hematology of University Hospital, Seattle, Wash., talked on "Coagulation Factors." His report included a lucid explanation of nomenclature.

Minoru Masuda, Research Assistant Professor in the Department of Psychiatry of University Hospital, discussed "Catecholamines and Metabolites" at the February meeting. He spoke briefly on the clinical significance and at length on methods of determining VMA.

A Symposium on lipids was held in March. Discussants were Edward Barron, Alex Kaplan, and Peter Ways. The discussion involved blood lipids, their physiological significance

and methods of determination. An interesting discussion of methodology followed.

## Clinical Chemistry Sessions at AAAS Meeting, Cleveland, Ohio

The American Association of Clinical Chemists will participate in the 131st Annual Meeting of the American Association for the Advancement of Science in Cleveland, Ohio, Dec. 26–31, 1963.

The tentative plan is to hold 3 half-day sessions for contributed papers. One of these sessions will be devoted to chromatographic and microchemical technics, provided a sufficient number of papers on these subjects is received.

Members and other interested persons are invited to participate in these sessions. Titles and 200-word abstracts, in triplicate, should be submitted not later than Sept. 30 to the program chairman, WILLARD R. FAULKNER, The Cleveland Clinic Foundation, Cleveland 6, Ohio.

## Minutes of the National Executive Committee

An interim meeting of the National Executive Committee was held in Atlantic City on Apr. 17, 1963.

The first major item of business was the report of the Legislation Committee. Several significant facts were discussed in the report. First, the past year has seen a considerable increase in the number of states in which bills of interest to the membership have been introduced. In still other states, rulings have been handed down which are yet to be tried in the courts. These matters center on efforts to license or regulate laboratories, with lesser attention paid to those who supervise them. Legal rulings attempt to apply omnibus definitions of the practice of medicine which we as chemists cannot accept. To retain an equitable voice in the discussion of these matters where they arise it is more important than ever to maintain watchdog groups to inform the national committee of local activity. While the committee will welcome and accept such information from any member, it feels obliged to issue its opinions through local section chairmen, in coordination with the National Secretary. Secondly, the Committee pointed out the problem of boards which are appointed or elected to oversee the provisions of laws. These boards and commissions operate on the basis of regulations which do not always seem in strict accordance with the provisions of the enabling act. Following some discussion of this problem the Finance Committee was empowered to support the institution of legal suits in defense of chemists' professional rights.

The Public Relations Committee report described a close liaison with the group in charge of the Fifth International Congress of Clinical Chemistry to provide the widest distribution of information in advance of its opening. In addition, it is preparing a brochure describing the nature of clinical chemistry for distribution to interested

persons. The posibility of a permanent traveling exhibit was discussed and approved. This would be made available to schools and other nonprofessional groups as the opportunity occurred.

The Membership Committee reported that since the new tighter requirements for membership have been in effect its problems have increased. Since January, 1963, it has processed 38 applications for membership; these have required 84 follow-up letters! These result from slow forwarding of applications by local sections, from the problem of reinstatement of lapsed memberships, and from the new requirement of academic transcripts from prospective associate and affiliate members. All local sections are urged to make an extra effort to assist the Committee in equitably and efficiently reviewing new applications.

The Ethics Committee raised the issue of membership in the Association by corporations or companies. Since our constitution makes no provision for any but personal memberships, such a practice is in violation of our Code of Ethics. The Committee was empowered to do whatever is necessary to prevent unethical action of this sort.

The delegate to the Intersociety Committee reported on a recent meeting of that body to the effect that little accomplishment can be expected pending some signs of activity by the counterpart agency of the American Medical Association.

The delegate to the American Association for the Advancement of Science urged that modest financial support be made available to reinforce our affiliation. He pointed out that the meetings are not always held in cities or areas where our local membership is strong. His suggestion was accepted and approved. Proper requests should in future be addressed to the Finance Committee.

Plans for the 1964 Annual Meeting in Boston were summarized by Dr. Ronan. The headquarter hotel will be the Statler-Hilton; other early plans are proceeding as expected.

Dr. Caraway indicated that the organization of the forthcoming International Congress is substantially complete. The four planned morning symposia will deal with the topics of Enzymes (planned as memorial tribute to Dr. E. J. King), Lipids, Metabolic Diseases, and Physical Methods. For the afternoon sessions of invited papers 120 manuscripts have already been received; reservations have been made by 40 exhibitors, and it appears that the only matter left is the registration of members, who are urged to complete and return their registration forms as soon as possible.

Dr. Sendroy spoke for the National Research Council Committee; he described efforts of that committee to increase the acceptance of doctorate training programs in academic circles. Means of increasing interest in postdoctoral training are being considered, and advisory statements are being formulated.

The Education Committee announced that is has been in consultation with officials of the National Institutes of Health concerning the prospect of training grants in clinical

chemistry. Particular interest has been expressed in placing training grants in departments of biochemistry which are associated with clinical laboratories. Those who wish further information are urged to contact the chairman of the Education Committee, Dr. Martin Rubin, at Georgetown University.

The Constitutional Review Committee presented a brief summary of its ideas on revision of executive committee structure. The advantages of a smaller steering group and a bicameral executive were discussed. This matter will be taken up again at the next annual meeting.

The Standards Committee has undertaken an ambitious program of activity, including continuation of bilirubin standardization, cholesterol standardization, and the preparation of turbidity standards. These problems are proceeding slowly because much of the work is done on a volunteer basis. Attempts to set up a system of standardized nomenclature and usage of units has been slowed by apparent disagreement concerning the desirable basis of the system. The committee hopes to establish a system of target priority for its future activities. A strong statement was made concerning the need for a uniform system of quality control; hopes exist that this may soon be established.

The Scholarship and Fellowship Committee reported on its talks with representatives of the Warner-Chilcott Company concerning their offer of a gift in support of a fellowship in clinical chemistry. Following recent directives of the Executive Committee matters have been brought to a head and it is hoped that announcements of the fellowship, to be known as the American Association of Clinical Chemists-Warner Chilcott Fellowship, may be made very soon. Announcements are in preparation for distribution to all approved schools and colleges, particularly to biochemistry departments. To be eligible, a candidate must have either the M.D. or M.S. degree, or the equivalent, and must be accepted by an approved graduate school. It is also stipulated that a candidate must submit a description of a research problem in which he is engaged and which is supervised by a recognized clinical chemist, preferably a certified clinical chemist. It is hoped that the first award of the fellowship may be made in time for the 1963-1964 academic year. Renewal for a second year will be contingent on performance of the candidate.

Harold Appleton next presented a report on the status of plans to increase the size of CLINICAL CHEMISTRY. This program was approved in principal at the last annual meeting. It is now clear that the best procedure will call for the adoption of a monthly format, which can be started as of January, 1964. To accomplish this will require an increased budget for office staff and for additional fixed equipment. The increased work load also will make it necessary for the Managing Editor to receive an honorarium to compensate for other duties which must be relinquished. The increased frequency of the Journal will also call for a $2.00 per year

increase in cost of subscriptions for members of the AACC. This seems reasonable in consideration of the extra material to be published. The Finance Committee was authorized to make available the necessary funds according to an approved budget as of July, 1963. This will allow for preparation of monthly publication beginning with the next volume. Dr. Appleton was unanimously re-elected as Managing Editor for a 5-year term, and he was also authorized to increase the size of the Editorial Board to 15 members as need arises. The term of Board members was extended to a period of 5 years, retrocative to the date of current appointments.

The Finance Committee presented a running quarterly statement. The sense of the report is that our financial position would not be endangered by the increased investment soon to be made in the Journal, as described above. In view of the increased frequency of publication of the Journal, and in view of changes in support not provided by the Association, the National Secretary had requested the Finance Committee to increase the support afforded for his office and that of the National Treasurer. This was discussed and approved. Since funds had already been budgeted for purchase of fixed equipment, the increase authorized is to be used to defray additional personal and secretarial expenses. The Committee had received a protest from the Puget Sound Section concerning the dues assessed against affiliate members. They asked that the matter be reviewed, and also indicated that requirement of a Journal subscription for this class of membership might be excessive. After some discussion this was referred to the Membership Committee for study and recommendations.

It was announced that the American Board of Clinical Chemistry has started a program of certification in the field of toxicological chemistry; a small number of individuals have already been certified. The question of Fellowship status for such persons was discussed, and approval was given to automatic extension of Fellowship status. The Secretary was directed to prepare the necessary certificates.

The subject of income from Annual Meetings was then discussed, as a result of which a new by-law was adopted to the effect that any deficit incurred at meetings would be underwritten by the national treasury, but any profits which accrue are now regarded as an asset of the national treasury. This was approved and adopted.

The meeting then adjourned.

## Fifth International Congress on Clinical Chemistry
## Detroit, Mich.
## Aug. 19-23, 1963

The Congress is organized by the American Association of Clinical Chemists and the Canadian Society for Clinical Chemistry under the authority of the International Federation of Clinical Chemistry and the Commission on Clinical Chemistry of the International Union of Pure and Applied Chemistry.

All scientific sessions of the Congress and the technical exhibition will be held in Cobo Hall. The reception and dinner of the Congress will be held in the Statler-Hilton Hotel.

Application forms or further information concerning the Congress can be obtained from the Congress Secretary, DR. DONALD G. REMP, Henry Ford Hospital, Detroit 2, Mich., U.S.A.

### Scientific Program

#### Monday Morning, Aug. 19

9:00– 9:25: Opening ceremony

*Symposium on Lipid Metabolism*

DR. D. B. TONKS, *Chairman*

9:25– 9:30 Introductory remarks

9:30–10:10 GEORGE CAHILL, JR.—Adipose tissue

10:10–10:30 Coffee break

10:30–11:10 R. ROSSITER—Metabolism and function of phospholipids and sphingolipids

11:10–11:50 R. R. TCHEN—Some aspects of cholesterol metabolism

11:50–12:30 R. J. HAVEL—The metabolism of circulating lipids

12:30– 1:00 Discussion (Opened by C. HOLLENBERG)

1:00– 2:00 Lunch

#### Afternoon, Aug. 19

Scientific Sessions (see below):

A, Automation

B, Metabolic (various)

C, Liver Dysfunction

D, Electrolytes

#### Tuesday Morning, Aug. 20

*Symposium on Enzymes in Clinical Chemistry* (PROF. E. J. KING Memorial). Organized by J. G. REINHOLD; DR. C. P. STEWART, *Chairman*

9:00– 9:15 Opening remarks and eulogy

9:15– 9:55 Y. HATEFI—Recent advances in electron transport and oxidative phosphorylation
9:55–10:35 L. K. YIELDING—Hormonal regulation of enzyme activity
10:35–11:00 Coffee break
11:00–11:40 B. HESS—Cellular enzymes in serum and their significance in terms of disturbed cell function
11:40–12:20 J. H. WILKINSON—Recent advances in clinical enzymology
12:20–12:45 Discussion (Opened by K. S. HENLEY)
12:45– 1:45 Lunch

### Afternoon, Aug. 20

Scientific Sessions (see below):
A, (1) Coagulation, (2) Atomic absorption
B, Quality control
C, Electrophoresis and chromatography
D, Hormones

### Thursday Morning, Aug. 22

*Symposium on Physical Methods in Clinical Chemistry*
B. ZAK, *Chairman*

9:00– 9:05 Opening remarks
9:05– 9:45 J. B. WILLIS–The analysis of biological materials by atomic absorption spectroscopy
9:45–10:30 ALBERT ZLATKIS–Affinity of organic compounds for free electrons with thermal energies
10:30–10:50 Coffee break
10:50–11:30 OTTO H. MILLER—Polarographic reactions of plasma proteins and their clinical significance
11:30–12:10 SAMUEL NATELSON—X-ray fluorescence spectrometry
12:10–12:30 Discussion
12:30– 1:30 Lunch

### Afternoon, Aug. 22

Scientific Sessions (see below):
A, Amino acid metabolism
B, Enzymes I
C, Ca, Mg and trace metals
D, Methods and normals

## Friday Morning, Aug. 23

*Symposium on Metabolism*
S. H. JACKSON, *Chairman*

9:00– 9:05 Opening remarks
9:05– 9:45 G. H. LATHE (Warner-Chilcott Lecturer)—The metabolism of bilirubin
9:45–10:30 OTTO WIELAND—Studies on the metabolism of isolated liver
10:30–10:50 Coffee break
10:50–11:30 R. J. WINZLER—The metabolism of glycoproteins
11:30–12:10 A. SASS-KORTSAK—The metabolism of copper
12:10–12:30 Discussion
12:30– 1:30 Lunch

## Afternoon, Aug. 23

Scientific Sessions (see below):
A, (1) Enzymes II, (2) Bilirubin
B, Carbohydrate
C, Proteins

## Scientific Sessions

### Monday, Aug. 19

*Section A: Automation*

2:00 No. 1. Methods for the automated determination of serum phosphate. E. EPSTEIN, E. S. BAGINSKI, and B. ZAK (Departments of Pathology, St. Joseph Mercy Hospital, Pontiac, Mich., Sinai Hospital, and Wayne State University College of Medicine. Detroit, Mich.).

2:15 No. 2. An automated procedure for the determination of phosphorus and alkaline phosphatase (Bodansky) in serum. NORBERT W. TIETZ and ALLEN GREEN (Mount Sinai Hospital, Chicago 8. Ill.).

2:30 No. 3. A photometric system for automatically reading concentration. D. K. MCKAY, D. SELIGSON, and W. R. JEWETT (Grace-New Haven Community Hospital and Yale University School of Medicine, New Haven 4, Conn.).

2:45 No. 4. Multiple automatic sequential analysis. I. Theoretical considerations. LEONARD T. SKEGGS (Department of Medicine and Surgery, Veterans Administration Hospital and Department of Pathology, Western Reserve University, Cleveland, Ohio.).

3:00 No. 5. Multiple automatic sequential analysis. II. Construction and operation of an eight channel instrument. LEONARD T. SKEGGS

(Department of Medicine and Surgery, Veterans Administration Hospital and Department of Pathology Western Reserve University, Cleveland, Ohio).

3:15 Coffee break

3:45 The effect of automation on the organization and operation of clinical laboratories. D. M. YOUNG (Director, Department of Laboratories, Toronto General Hospital, Toronto, Canada).

*Section B: Metabolic (various)*

2:00 No. 6. Uptake of aromatic amines by blood platelets. D. V. SIVA SANKAR and D. BARBARA SANKAR (Creedmoor State Hospital, Jamaica 27, N. Y.).

2:15 No. 7. Mauve factor and 6-sulfatoxy-skatole: Two biochemical abnormalities associated with specific measures of psychiatric disease. D. G. IRVINE (Research Biochemist, Saskatchewan Hospital, North Battleford, Sask., Canada).

2:30 No. 8. Serum lipids and cancer. HARALD VON ELMENDORFF (Dusseldorf, Medizinische Akademie, Moorenstrasse 5, Germany).

2:45 No. 9. The effect of X-ray irradiation on the desoxyribonucleic acids of the spleen. H. P. SCHWARZ, H. DESHER, M. HAINSKI, and R. LUMLEY JONES (Biochemistry Laboratory, Philadelphia General Hospital, Philadelphia, Pa.).

3:00 Coffee break

3:30 No. 10. Surgical trauma and arylsulphatase activity of blood and the healing wound. L. M. DZIALOSZYNSKI, J. NOVAK, and A. FROHLICH (Animal Physiology Department, College of Agriculture, Poznan, Poland).

3:45 No. 11. Graded pilocarpine tests in the study of cystic fibrosis. A. L. TARNOFFY (Royal Berkshire Hospital, Reading, England).

4:00 No. 12. Das auffinden eines neuen mono-pyrrols (porphobilinogen B) in porphyriaurinin. H. PETERMANN and J. WYLLIE (Department of Chemistry, Queen's University, Kingston, Ontario).

4:15 No. 13. The induction of porphyria in rats by hexachlorobenzene administration. J. WYLLIE and H. PETERMANN (Department of Chemistry, Queen's University, Kingston, Ontario).

*Section C: Liver Dysfunction*

2:00 No. 14. Studies on severe familial idiopathic jaundice of infancy. O. P. GRAY and R. A. SAUNDERS (The United Cardiff Hospitals, Clinical Pathology, Llandough Hospital, Penarth, Glam, Wales).

2:15 No. 15. Liver mitochondria as a source of plasma enzymes. MAX M. FRIEDMAN (Chemistry Division and the Dr. Milton J. Good-

friend Laboratory, Department of Laboratories, Bronx-Lebanon Hospital Center, Concourse Division, Bronx, N. Y.).

2:30 No. 16. A comparison of the serum L.A.P. with the hepatoscan technique in the investigation of liver metastases. J. B. DERRICK, F. C. HEAGY, R. URTASUM, and D. P. SWARTZ (Departments of Medical Research, Obstetrics and Gynecology and Radio Therapy, University of Western Ontario, London, Ont., Canada).

2:45 No. 17. N-acetyl-β-glucosaminidase activity of the serum and liver in hepatic damage. MASAHISA WADA and NATSUKI MITSUTANI (University Hospital, First Department of Internal Medicine, Osaka University, Osaka, Japan).

3:00 No. 18. Serum adenosine deaminase in the jaundiced patient. D. M. GOLDBERG (Department of Biochemistry, Western Infirmary, Glasgow, Scotland).

3:15 Coffee break

3:45 No. 19. Histidase and urocaninase of the blood in liver lesions. S. R. MARDASHEV (Department of Biochemistry, The First Moscow Medical Institute, Moscow, U.S.S.R.).

4:00 No. 20. A rapid modification of the cephalin cholesterol flocculation test. ARTHUR A. ROSENBERG, LUCILLE G. EIMANN, and JOHN O'LEARY (Laboratory Service, Veterans Administration Hospital, Albany, N. Y.).

4:15 No. 21. Qualitative changes of human plasma proteins in cirrhosis of the liver. T. R. NIEDERLAND (III. Medical Clinic; Research Laboratory of Pharmobiochemistry, Komensky University Medical School, Hiboka II, Bratislava, Czechoslovakia).

*Section D: Electrolytes*

2:00 No. 22. Normal ranges of acid-base control. JOHN M. HARRELSON and RALPH E. THIERS (Clinical Chemistry Laboratory, Duke University Medical Center, Durham, N. C.).

2:15 No. 23. Quantitative aspects of acid-base balance. RALPH E. THIERS and JOHN M. HARRELSON (Clinical Chemistry Laboratory, Duke University Medical Center, Durham, N. C.).

2:30 No. 24. Ultramicrotechnique for the determination of total bases of plasma. F. TEIXEIRA, A. MARTINS, and F. MOURA PREZA (Laboratory of the Department of Pediatrics, Lisbon University, Faculty of Medicine, Lisbon, Portugal).

2:45 No. 25. Practical suggestions for the determination of sodium and potassium in serum. BERTIL JOSEPHSON, JONAS BERGSTROM, and ERIC HULTMAN (Central Clinical Laboratory, St. Erik's Hospital, Stockholm, Sweden).

3:00 No. 26. Activation analysis of tissue electrolytes. JONAS BERGSTROM (The Central Clinical Laboratory and the Renal Clinic, St. Erik's Hospital, Stockholm, Sweden).

3:15 Coffee break

3:45 No. 27. Metabolic studies in a patient with aldosterone-secreting tumor. JAMES C. EATON (University Department of Pathological Biochemistry, The Royal Infirmary, Glasgow, Scotland).

4:00 No. 28. The determination of fluorine in urine by microdiffusion. L. DUBOIS, T. TEICHMAN, and J. L. MONKMAN (Department of National Health and Welfare, Occupational Health Division, Ottawa 4, Canada).

4:15 No. 29. Gasometric analysis of carbon monoxide in air. JULIUS SENDROY, JR. and JOHN D. O'NEAL (Division of Chemistry, U. S. Naval Medical Research Institute National Naval Medical Center, Bethesda, Md.).

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## Tuesday, Aug. 20

*Section A: Coagulation*

1:45 No. 30. Kinetics and distribution of $I^{131}$ labeled rabbit anti-human fibrinogen in man. DAVID MARRACK, WILLIAM C. DEWEY, WILLIAM F. BALE, and RAYMOND G. ROSE (Departments of Pathology, Physics and Medicine, The University of Texas M.D. Anderson Hospital and Tumor Institute, Houston, Tex., and Department of Radiation Biology, University of Rochester School of Medicine and Dentistry, Rochester, N. Y.).

2:00 No. 31. An evaluation of techniques for separation and estimation of plasma fibrinogen. JESSE F. GOODWIN (General Clinical Research Center, Wayne State University College of Medicine, Detroit 2, Mich.).

2:15 No. 32. A dye-uptake technique for plasma fibrinogen quantitation. J. A. FOREMAN, G. L. DOUGLAS, L. M. BERGQUIST, and R. L. SEARCY (Departments of Pathology, Los Angeles County General Hospital (Unit 2), and California College of Medicine, Los Angeles, Calif.).

2:30 No. 33. Blood lipids in relation to coagulation. N. F. MACLAGAN and J. D. BILLIMORIA (Department of Chemical Pathology, Westminster Medical School, London S.W. 1, England).

2:45 No. 34. Standardization of the one-stage "prothrombin time" assay. ALAN MATHER (The Memorial Hospital, Wilmington, Del.).

3:00 No. 35. Laboratory control of anti-coagulant therapy. S. WINSTEN

(Department of Laboratories, Albert Einstein Medical Center, Philadelphia 41, Pa.).

3:15 Coffee break

*Atomic Absorption*

3:30 No. 36. Estimation of magnesium by atomic absorption spectrophotometry. D. B. HORN and A. L. LATNER (Department of Clinical Chemistry, The Medical School and Royal Victoria Infirmary, Newcastle-upon-Tyne, England).

3:45 No. 37. The determination of serum calcium using atomic absorption spectrophotometry. D. SELIGSON and A. ZETTNER (Grace-New Haven Community Hospital and Yale University School of Medicine, New Haven 4, Conn.).

4:00 No. 38. An application of atomic absorption spectrometry in clinical chemistry: determination of copper in biological materials. ELEANOR BERMAN (Illinois Masonic Hospital Association, Chicago 14, Ill.).

4:15 No. 39. Determination of trace metals in biological materials by atomic absorption spectrophotometry. WALTER SLAVIN (Perkin-Elmer Corporation, Norwalk, Conn.).

*Section B: Quality Control*

1:45 No. 40. Biological versus analytical errors. ROBERT P. HARPUR (Institute of Parasitology, McGill University, Macdonald College, P. O., Quebec, Canada).

2:00 No. 41. Quality control in clinical chemistry. I. Description of an intermedical laboratory quality analysis survey. C. J. PORTER (Department of Laboratories, Toronto General Hospital, Toronto, Canada).

2:15 No. 42. Quality control in clinical chemistry. II. Application of the information obtained from an intermedical laboratory quality analysis survey. D. M. YOUNG (Department of Laboratories, Toronto General Hospital, Toronto, Canada).

2:30 No. 43. The reliability of clinical chemistry determinations; computer analysis of day to day variability. MARTIN H. MURRAY and ANTHONY J. LAPAGLIA (Departments of Chemistry, Genesee Hospital and Highland Hospital, Rochester, N. Y.).

2:45 No. 44. Evaluation of serum preparations used for controlling total cholesterol measurements. L. M. BERGQUIST, J. A. GIDDINGS, and R. L. SEARCY (Departments of Pathology, Los Angeles County General Hospital (Unit 2) and California College of Medicine, Los Angeles, Calif.).

3:00 No. 45. The use of probability paper in analysing frequency distributions. D. H. CURNOW and KEITH SHEARD (Royal Perth Hos-

pital and University of Western Australia, Perth, Western Australia).

*Section C: Electrophoresis and Chromatography*

1:45 No. 46. Effects of tetracyclines on electrophoretic mobilities of serum lipid-bearing proteins. R. L. Searcy, J. A. Foreman, and L. M. Bergquist (Departments of Pathology, Los Angeles County General Hospital (Unit 2) Los Angeles, Calif.).

2:00 No. 47. Electrophoresis in synthetic gel. II. Molecular vs. gel factors. Samuel Raymond, Masumi Nakamichi, and Ina Frank (Pepper Laboratory of Clinical Medicine, School of Medicine, University of Pennsylvania, Pa.).

2:15 No. 48. Electrophoretic separation of RNA nucleotides on agar gel-paper. B. Zak and L. M. Weiner (Departments of Pathology and Microbiology, Wayne State University College of Medicine, Detroit, Mich.).

2:30 No. 49. A rapid quantitative method for concentrating urine for protein electrophoresis. Hylton McFarlane (University of the West Indies, Department of Pathology, Kingston 7, Jamaica).

2:45 No. 50. A new method of recording the light absorption of the eluate of a chromatography column. Denis Boutin and Eleanor Harpur (Laboratories, Hospital St. Joseph, LaTuque, Quebec, and the Department of Biochemistry, The Montreal Children's Hospital, Montreal, Canada).

3:00 No. 51. Urea, a new stain in organic acids paper chromatography. M. J. Halpern (Department of Physiological Chemistry, Faculty of Medicine, Lisbon, Portugal).

3:15 Coffee break

3:45 No. 52. Chromatographic studies of the carbohydrate components of the human gastric mucopolysaccharides. J. Schrager (Department of Pathology, Royal Albert Edward Infirmary, Wigan, England).

4:00 No. 53. The determination of arginase activity by means of paper chromatography. R. P. Barschel (University of Saskatchewan, Regina Campus, Regina, Sask., Canada).

4:15 No. 54. A preparative technique for isolation of materials of relatively high volatility for gas chromatographic quantitation. Richard H. Gadsden and William M. McCord (Department of Chemistry, Medical College of South Carolina, Charleston, S. C.).

*Section D: Hormones*

1:45 No. 55. Fractionation of urinary corticosteroids in healthy individuals and in patients with liver disease. Inge Dyrenfurth

and LIONEL E. MCLEOD (Department of Medicine, University of Alberta Hospital, Edmonton, Alta., Canada).

2:00 No. 56. Evaluation of procedures for urinary 17-hydroxycorticosteroids. W. RAGLAND BROWN (Bio-Assay Laboratory, Dallas, Tex.).

2:15 No. 57. The determination of urinary estrogens. DAVID J. WATSON, JOE M. BURKEY, and PAIGE K. BESCH (Steroid Research Laboratories, Department of Obstetrics and Gynecology, Ohio State University, College of Medicine, Columbus 10, Ohio).

2:30 No. 58. Placental transfer of a new progestin. PAIGE K. BESCH, ROBERT E. BAKER, and SIBU GHOSH (Steroid Research Laboratories, Department of Obstetrics and Gynecology, Ohio State University, College of Medicine, Columbus 10, Ohio).

2:45 No. 59. The rapid determination of cortisol and thyroxine in plasma using gel filtration. BEVERLY PEARSON MURPHY and CHAUNCY J. PATTEE (Clinical Investigation Unit, Queen Mary Veterans' Hospital, Montreal, Canada).

3:00 No. 60. Interference in the determination of urinary catecholamines by formaldehyde forming drugs. H. RICHTER and MARY MEUFFELS (Department of Biochemistry, Ottawa Civic Hospital, Ottawa 3, Canada).

3:15 Coffee break

3:45 No. 61. The serotonin content of platelets in pulmonary arterial and venous blood sampled during thoracic surgery. NEVILLE CRAWFORD (Cleveland Clinic, Research Division, Cleveland 6, Ohio).

4:00 No. 62. The multiplicity of antibodies against insulin. MASAYASU KITAGAWA and YUICHI YAMAMURA (Department of Biochemistry, School of Medicine, Kyushu University, Fukuoka and 3rd Department of Internal Medicine, Osaka University Hospital, Osaka, Japan).

4:15 No. 63. Serum-$B_{12}$ post resectionem ventriculi. ERIC HAMMERICH DAMM (The Central Laboratory, Skive Sygehus, Denmark).

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## Thursday, Aug. 22

*Section A: Amino Acid Metabolism*

1:30 No. 64 Studies on isovalthine. SHUNZI MIZUHARA (Department of Biochemistry, Okayama University Medical School, Okayama, Japan).

1:45 No. 65. The photometric determination of the tryptophan content of

proteins hydrolyzed with perchloric acid. ABRAHAM SAIFER and SHIRLEY GERSTENFELD (Isaac Albert Research Institute of the Jewish Chronic Disease Hospital, Brooklyn 3, N. Y.).

2:00 No. 66. Tryptophan metabolism in patients with infantile spasms. P. HUGHES and B. D. BOWER (Children's Hospital, Birmingham, England).

2:15 No. 67. Tryptophan metabolism in rheumatoid arthritis. ISOBEL M. BETT (Rheumatic Diseases Unit, Northern General Hospital, Ferry Road, Edinburgh 5, Scotland).

2:30 No. 68. A study on host-tumor relationship with special reference to amino acid nutrition and protein metabolism. MASAMI SUDA (Division of Protein Metabolism, Institute for Protein Research, Osaka University, Osaka, Japan).

2:45 No. 69. Detoxicating effect of L-aspartic acid, L-ornithine hydrochloride and L-ornithine L-aspartate with special reference to the ammonia level in serum. KYUJI ABE, YOSHIO KOWA, SHOICHI HARIGAYA, and TAKASHI MORITA (Tokyo Research Laboratory, Tanabe Seiyaku Co., Ltd., Saitamaken, Japan).

3:00 No. 70. The influence of age on the amino acid level in blood plasma. GEORGE T. LEWIS, RICHARD F. WEHR, VALENTINE MOORE (Department of Biochemistry, University of Miami School of Medicine, Miami, Fla.).

3:15 Coffee break

3:45 No. 71. A routine procedure for screening of phenylketonuria in the newborn. PAUL WONG, TOHRU INOUYE, and DAVID YI-YUNG HSIA (Department of Pediatrics, Northwestern University Medical School and Children's Memorial Hospital, Chicago, Ill.).

4:00 No. 72. Glutathione in anemias. NIKO JESENOVEC and MARIJANA FISER-HERMAN (Institute for Clinical Chemistry, Zagreb, Domagojeva, 2.I., Yugoslavia).

4:15 No. 73. Chromatographic studies of amino acids in sera and urine of patients with xeroderma pigmentosum and their normal relatives. H. EL-HEFNAWI and M. F. S. EL-HAWARY (Faculty of Medicine, Cairo University and National Research Centre, Nutrition Unit, Dokki, Cairo, U.A.R.).

*Section B: Enzymes I.*

1:30 No. 74. Some clinical experience with isoenzymes. A. L. LATNER and A. W. SKILLEN (Department of Clinical Chemistry, The Medical School and Royal Victoria Infirmary, Newcastle-upon-Tyne, England).

1:45 No. 75. Electrophoretic and other techniques in the study of serum

isoenzymes after myocardial infarction. A. L. LATNER and A. W. SKILLEN (Department of Clinical Chemistry, The Medical School and Royal Victoria Infirmary, Newcastle-upon-Tyne, England).

2:00 No. 76. The experimental and clinical studies on β-glucuronidase isozymes. Y. YAMAMURA, T. AOKI, T. OKOCHI, Y. TAKAHASHI, and F. ITO (The Third Department of the Internal Medicine, University of Osaka School of Medicine, Osaka, Japan).

2:15 No. 77. Plasma N-acetyl-β-glucosaminidase and β-glucuronidase in health and disease. J. W. WOOLLEN and P. TURNER (Biochemistry Department, Royal National Orthopaedic Hospital, Stanmore, Middlesex and Edgware General Hospital, Edgware, Middlesex, England).

2:30 No. 78. Studies on the alkaline phosphatase of human urine. D. W. MOSS, P. J. BUTTERWORTH, and E. PITKANEN (Department of Chemical Pathology, Postgraduate Medical School, London, W.12, England).

2:45 No. 79. Urinary excretion of alkaline phosphatase in normal and cirrhotic persons. ALBERT A. DIETZ (Veterans Administration Hospital, Albany, N. Y.).

3:00 No. 80. La trehalase du serum sanguin. JEAN EMILE COURTOIS, MARIE MADELEINE DEBRIS, and JEAN CLAUDE GEORGET (Laboratoire de Chimie Clinique de la Pharmacie de l'Hopital Laennec, Paris, France).

3:15 Coffee break

3:45 No. 81. The clinical significance of synovial fluid acid phosphatase levels. JACOB KREAM, MARTIN A. LEHMAN, CATHERINE KAMBOLIS, and DONATO BROGNA (The Departments of Biochemistry and Pathology of the Laboratory Division and the Department of Orthopaedic Surgery, Hospital for Joint Disease, New York, N. Y.).

4:00 No. 82. Lactic dehydrogenase isoenzymes in normal and disease states. J. C. NIXON and G. F. FITZGIBBON (National Defense Medical Centre, Ottawa, Canada).

4:15 No. 83. On the proteinases of malignant tumors. V. N. OREKOHVICH and O. V. KOUNINA (Institute of Biological and Medical Chemistry, Academy of Medcial Sciences of the U.S.S.R., Pogodin St. 10, Moscow G-117, U.S.S.R.).

4:30 No. 84. LDH isoenzymes. R. J. WIEME (Interne Kliniek Akademisch Ziekenhuis, De Pintelaan Ghent, Belgium).

*Section C: Calcium, Magnesium, and Trace Metals*

1:30 No. 85. Determination of calcium-ion concentrations in the serum or

plasma for clinical routine work. LEVI M. MARIO and ELAZAR M. SILVIO (Biochemical Laboratory of the Institute of Hygiene and Social Medicine, Medical School University of Sarajevo, Yugoslavia).

1:45 No. 86. A simple photometric instrument for the microdetermination of serum calcium. KURT M. DUBOWSKI (Department of Biochemistry, University of Oklahoma School of Medicine, Oklahoma City 4, Okla.).

2:00 No. 87. A new EDTA method for the determination of calcium in blood serum. WILLIAM RICHARD DOUGLAS (Finseninstitut ag Radium stationen, Copenhagen Postboks 67, Copenhagen K, Denmark).

2:15 No. 88. Etude de la citraturie, particulierement dans la lithiase renale. P. DESGREZ, J. THOMAS, E. THOMAS, and H. RABUSSIER (*Laboratoire de Chimie, Faculte de Medecine, 45, rue des* Saints-Peres, Paris 60, France).

2:30 No. 89. The role of citrate in the metabolism of divalent cations. SAMIR HANNA (Clinical Pathology Department, Faculty of Medicine, Ein Shams University, Abbassiah, Cairo, U.A.R.).

2:45 No. 90. Plasma magnesium in children. SAMIR HANNA (Clinical Pathology Department, Faculty of Medicine, Ein Shams University, Abbassiah, Cairo, U.A.R.).

3:00 Coffee break

3:30 No. 91. Normal occurrence of selenium in food and body tissues. H. H. TAUSSKY and A. T. MILHORAT (Institute for Muscle Disease, Inc., New York 21, N. Y.).

3:45 No. 92. Determination of microgram quantities of copper in biological material. RAMON STONER and WALDEMAR DASLER (Department of Biochemistry, The Chicago Medical School, Chicago, Ill.).

4:00 No. 93. An improved serum iron measurement with sulfonated bathophenanthroline. ROGER CALLAHAN (Research and Medical Services, Veterans Administration Hospital, Oklahoma City, Okla., and Department of Medicine, University of Oklahoma School of Medicine).

4:15 No. 94. Determination of iron in serum using solvent extraction. D. T. FORMAN, H. A. WOODSON, and S. JOCHER (Department of Pathology, Mercy Hospital, and Department of Biochemistry, Northwestern University Medical School, Chicago, Ill.).

*Section D: Methods and Normals*

1:30 No. 95. Recent concepts in clinical microchemistry. SAMUEL MEITES (Children's Hospital, Columbus 5, Ohio).

1:45 No. 96. Determination of creatine and creatinine in urine. ANDRE C.

KIRBRICK and ADE T. BILHORAT (Institute for Muscle Disease, New York, N. Y.).

2:00 No. 97. The urinary excretion of creatine and creatinine by children in hospital. S. H. JACKSON and D. M. MILLS (Research Institute, The Hospital for Sick Children, Toronto, Canada).

2:15 No. 98. Age variations of NPN and urea of newborn infants. BERTIL JOSEPHSON and OLLE JARNMARK (Central Clinical Laboratory, St. Erik's Hospital, Stockholm Sweden).

2:30 No. 99. Plasma P.S.P. index of renal function. M. HENRY GAULT, T. DOUGLAS KINSELLA, and B. KOCH (Department of Clinical Chemistry, Queen Mary Veterans' Hospital and Departments of Endocrinology and Metabolism and Urology, Royal Victoria Hospital, Montreal, Canada).

2:45 No. 100. Cholesterol and phospholipid in human cerebrospinal fluid. YUNG SOO SHIN (*Department of Clinical Pathology, St. Mary* Mercy Hospital, Gary, Ind.).

3:00 Coffee break

3:30 No. 101. Enzymatic determination of glycerol in the measurement of triglycerides. F. H. KREUTZ (Med. Univ. Klinik, Marburg, Germany).

3:45 No. 102. Simultaneous determination of serum barbiturates and salicylates by ultra-violet spectrophotometry. SOLVEIG BJERRE and C. J. PORTER (Division of Chemistry and Biochemistry, Department of Laboratories, Toronto General Hospital, Toronto, Canada).

4:00 No. 103. Interference of thiouracil in the Zak cholesterol method and elimination of this interference with silver iodate. EUGENE W. RICE (Siner Research Laboratory, Allegheny General Hospital, Pittsburgh, Pa.).

4:15 No. 104. Gas chromatographic determination of 17 ketosteroids in urine—studies of normal human subjects. E. H. KEUTMANN, MARIO SPARAGANA, *and* WILLIAM B. MASON (*School of Medicine and Dentistry, University of Rochester, Rochester 20, N. Y.*).

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## Friday, Aug. 23

*Section A: Enzymes II.*

1:30 No. 105. Studies on the enzyme secretion of the human pancreas. W. RICK (Medizinische Universitatsklinik, Giessen, Germany).

1:45 No. 106. Lipase-tributyrinase of breast milk and gastric digestion of newborns. B. M. SOUMTSOV (Biochemical Laboratory, SRIOG, Moscow G-2, Plotniken, per 7, ap. 2 U.S.S.R.).

2:00 No. 107. Separation of amylase from human serum and urine by gel filtration and the demonstration of an abnormal binding of serum amylase by a γ-globulin. P. Wilding, W. T. Cooke, and R. Gaddie (General Hospital, Birmingham, England).

2:15 No. 108. Electrophoretic distribution of amylase activity. J. Edward Berk, Matsamitsu Kawaguchi, Ichiro Ujihira, Rashid Zeineh, and Ronald L. Searcy (Departments of Medicine and Pathology; Los Angeles County General Hospital (Unit 2) and California College of Medicine, Los Angeles, and Department of Medicine, Sinai Hospital of Detroit and Wayne State University College of Medicine, Detroit, Mich.).

2:30 No. 109. Cholinesterase activities. Marjorie Bick (Alfred Hospital, Melbourne, Australia).

2:45 No. 110. Glucose-6-phosphatase deficiency on Curacao (Neth. Ant.) H. Schouten and A. Van der Sar (St. Elisabeth Hospital, Breedestraat 193 (0) Willemstad, Curacao N.A.).

3:00 No. 111. Isolation and properties of an esterase from hog liver microsomes. Klaus Krisch (Physiologisch-chemisches Institute der Universitat, Giessen, Germany).

3:15 Coffee break

*Bilirubin*

3:30 No. 112. Different effects of light on bilirubin of jaundiced serum. S. H. Blondheim and N. Kaufman (Metabolic Unit and Laboratory, Hadassah University Hospital, Jerusalem, Israel).

3:45 No. 113. The bilirubin-binding-capacity (BBC) of blood. Derek Watson, Diane D. Nankville, and Mun H. Own (Department of Biochemistry, The Royal Women's Hospital, Carlton N. 3, Victoria, Australia).

4:00 No. 114. The ante partum diagnosis of hemolytic disease by amniotic fluid bilirubin analysis. A. G. Stewart (Department of Paediatrics, University of Alberta Hospital, Edmonton, Alta., Canada).

4:15 No. 115. Studies on the determination of urobilinogen in urine as urobilinogen-aldehyde. Alberto A. Fernandez, Richard J. Henry, and Sam Berkman (Bio-Science Laboratories, Los Angeles 25, Calif.).

*Section B: Carbohydrates*

1:30 No. 116. Clinical applications of infrared spectroscopy. III. Identification of sugars. Bernard Klein and Milton Weissman (Biochemistry Laboratory, Veterans Administration Hospital, Bronx 68, N. Y.).

1:45 No. 117. Enzymatic determination of glucose in urine following rapid

removal of inhibitors by ion-exchange resin. J. E. LOGAN and D. E. HAIGHT (Clinical Laboratories Laboratory of Hygiene, Department of National Health and Welfare, Ottawa, Canada).

2:00 No. 118. Studies with a simplified nitroprusside test for ketone bodies in urine and serum. JOSEPH FRASER, MARION C. FETTER, RAYMOND L. MAST, and ALFRED H. FREE (Ames Research Laboratory, Elkhart, Ind.).

2:15 No. 119. Screening tests for cerebrospinal fluid glucose. HELEN M. FREE and JEANNE A. WASMUNDT (Ames Research Laboratory, Elkhart, Ind.).

2:30 No. 120. A study of the Momose fluorometric determination of blood glucose. BERNADETTE B. BOURNE (Departments of Neurology and of Biochemistry, University of Miami, Miami, Fla.).

2:45 No. 121. Study of some enzymes of hexose monophosphate shunt in diabetics. J. A. LOPES DO ROSARIO (Laboratorio de Quimica Fisiologica, Faculdade de Medicine, Lisboa, Portugal).

3:00 No. 122. The concentration of glucose and other reducing substances in different organs of fed and fasting rats. J. KRAWCZYNSKI, R. VRBA, and H. S. BACHELARD (Department of Laboratory Diagnosis, Postgraduate Medical School in Warsaw and Neuropsychiatric Research Unit, Medical Research Council, Carshalton, Surrey, England).

3:15 Coffee break

3:45 No. 123. The pentose metabolic pathway in neoplastic disease. Y. S BROWNSTONE (Department of Pathological Chemistry, University of Western Ontario, London, Ont., Canada).

4:00 No. 124. The glycogen content of muscle tissue obtained by needle biopsy on normal and diabetic man. ERIC HULTMAN and JONAS BERGSTROM (The Central Clinical Laboratory, St. Erik's Hospital, Stockholm, Sweden).

4:15 No. 125. Technique and interpretation of continuous intravenous glucose recordings. EINHART KAWERAU and S. J. SURTUS (Department of Chemical Pathology, St. James' Hospital, Sarsfeld Road, Balham S.W. 12, England).

*Section C: Proteins and Glycoproteins*

1:30 No. 126. The fluorometric determination of albumin in cerebrospinal fluid and serum. ALEX KAPLAN and MURRAY A. JOHNSTONE (Department of Biochemistry, University of Washington, Seattle 5, Washington).

1:45 No. 127. The detection of abnormal proteins in plasma and serum by viscometry. JOHN HARKNESS and R. B. WHITTINGTON (South

Somerset Clinical Area Pathology Service Taunton and Somerset Hospital, Musgrove Park Branch, Taunton, England).

2:00 No. 128. Serum alpha-2 macroglobulin in children in health and disease. M. S. DEWOLFE (Research Institute, The Hospital for Sick Children, Toronto, Canada).

2:15 No. 129. Starch gel electrophoresis patterns of serum proteins in cystic fibrosis of the pancreas. ELEANOR R. HARPUR, ANNE H. MURDOCK and MIMI M. BELMONTE. (The Montreal Children's Hospital, Montreal, Quebec).

2:30 No. 130. Serum polysaccharides in transplantable and induced tumors of mice. YASIN ABD EL-CHAFFAR (Abbasiah Faculty of Medicine, Cairo, U.A.R.).

2:45 No. 131. Action of α-lipoic acid on ceruloplasmin and transferrin in several illnesses. S. F. GOMES DA COSTA, J. A. FILIPE DA SILVA and FELISBELA E. S. MALDONADO (Faculdade de Medicina de Lisboa, Portugal).

3:00 No. 132. Polarographic determination of fucose in serum proteins and protein-containing tissue extracts. Z. STARY, A. H. WARDI and D. L. TURNER (Warner State Hospital, Warren, Pa.).

3:15 Coffee break

3:45 No. 133. The effects of age and sex on the electrophoretic fractions of the serum glycoproteins. R. H. PEARCE, E. M. WATSON, R. STODOLSKI, J. M. MATHIESON and J. J. THEORET (Department of Pathological Chemistry, Faculty of Medicine, University of Western Ontario, London, Canada and Department of Pathology, Faculty of Medicine, University of British Columbia, Vancouver, Canada).

4:00 No. 134. Biochemical studies on mucopolysaccharides in synovial fluid. H. GREILING (Rheumaforeschungsinstitut beim, Landesbad, Aachen, Neth.).

4:15 No. 135. Studies on serum and liproproteins in normal Egyptians and in patients with medical kidney diseases. EZZAT ABDEL WAHHAB (Clinical Pathology, Faculty of Medicine, Ein Shams University, Cairo).

*Read by title*

No. 136. Effects of exceremental odor on growth-promoting substances. TOYOJI YORITAKA, MASAO FUJIMOTO and IKUO MUTA (Department of Biochemistry, School of Medicine, Nagasaki University, Japan).

# Serum Lactic Dehydrogenase Activity: An Analytical Assessment of Current Assays

*Elias Amador, Lionel E. Dorfman, and Warren E. C. Wacker*

**Serum LDH activities assayed spectrophotometrically by the "forward" (lactate + DPN to pyruvate + DPNH + H) method have linear reaction rates, activity proportional to the amount of serum added, and a reproducibility of 1.3%. The reagent mixture can be stored frozen for 6 months and in the lyophilized state indefinitely.**

**Poor separation of normal from abnormal serum LDH activities results from the nonlinear calibration curves and decreasing reaction rates of the "backward" spectrophotometric assay. The dinitrophenylhydrazine colorimetric assay is deemed unsuitable for clinical use because large variations in activity result from minor variations in the reagent calibration curve, and because colorimetric activities are inaccurate as compared to activities obtained with the "forward" spectrophotometric assay.**

THE DETERMINATION of serum lactic dehydrogenase activity (LDH) has achieved an important role as a positive diagnostic measure in suspected myocardial infarction. Serum LDH activity can be used safely both to confirm and to exclude this diagnosis (*1*). Such crucial decisions can be made safely only when the procedure employed for this assay is simple, reproducible, and, hence, capable of differentiating with certainty between normal and abnormal activities. The procedures currently employed for the assay of serum LDH activity have been evaluated in this context. The spectrophotometric method measuring the rate of reduction of DPN in the presence of lactate (*2,3*) fulfills these exacting criteria. Both the spectrophotometric method meas-

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ing the rate of DPNH oxidation in the presence of pyruvate (*4, 5*) and the dinitrophenylhydrazine colorimetric method (*6*) have been found wanting.

## Reagents

*Buffered solution of sodium pyrophosphate (0.05M, pH 8.8), lactic acid (77.5mM), and diphosphopyridine nucleotide (5.25mM) for "forward reaction"* Sodium pyrophosphate, 6.2 gm., is dissolved in 250 ml. of hot distilled water. The solution is cooled and lactic acid, 2.0 ml., is added; *p*H is adjusted to 8.8 with 1N NaOH. Diphosphopyridine nucleotide (DPN), 1.10 gm., is dissolved in this solution, and the volume is adjusted to 280 ml., after which 2.8-ml. aliquots are stored at −20°.* The mixture is stable for 6 months.

Sodium pyrophosphate was obtained from Baker Chemical Co. (Phillipsburg, N. J.) lactic acid from Merck and Company (Rahway, N. J.), and DPN from Pabst Laboratories (Milwaukee 5, Wis.). All chemicals were reagent grade. Stability of reagents was determined daily by assaying an aliquot of serum of known activity. These aliquots were prepared once a month and stored at −20°.

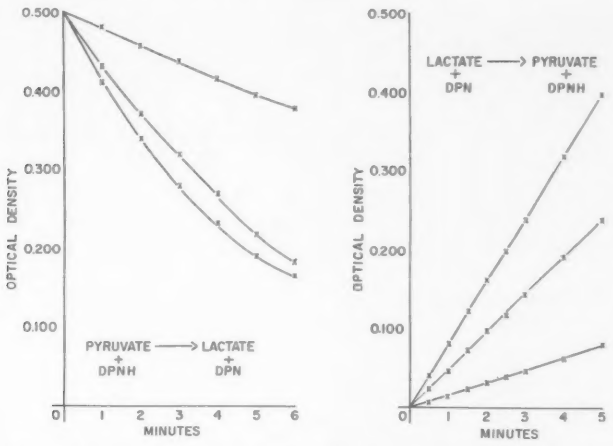
The reagents for the spectrophotometric assay of Kubowitz and Ott (*4*), as used by Wroblewski—the "backward reaction"—were prepared according to the technic of Henry *et al.* (*5*). Reagents obtained from the Sigma Chemical Co. (St. Louis, Mo.) were used in the colorimetric serum LDH assay following the procedure of Berger and Broida (*6*). The purity and stability of DPNH solutions were determined as previously described (*7*).

## Collection of Serum

Venipuncture is performed with a No. 20 needle. The blood is allowed to clot at room temperature, and the clot is gently separated from the test tube with an applicator stick. The blood is centrifuged for 10 min. at 2000 rpm, and then the serum is decanted and recentrifuged for 5 min. at 2000 rpm. Sera with visible hemolysis were discarded.† No significant rise in LDH activity occurs if clotted blood stands for 5 hr. at 25° or overnight at 4°.

*Lyophilized reaction mixtures are available as "LDH Determatubes" from the Worthington Biochemical Corp., Freehold, N. J.

†No significant elevations of serum LDH activity occurred when hemoglobin concentrations below 0.1 gm./100 ml. of serum were present. Hemoglobin concentrations above this value can be detected visually and produce spurious elevations of LDH activity.




1. Rates of reaction for serum LDH activity, shown as function of total activity, are [illegible] for DPNH-to-DPN assay (at left). With DPN-to-DPNH method described in text, rate of reaction are linear for activities with change of absorbance up to 0.100/min. (at right). Same serums were employed for both assays.

## Method

Tubes containing the 2.8 ml. of the buffered substrate mixture are thawed and incubated in a constant-temperature water bath* at 25°. Serum, 0.2 ml., is added to each tube and mixed by gentle inversion. The contents are transferred immediately to a 3-ml. Beckman cuvette of 1-cm. path length; absorbance increase at 340 mμ is recorded every 30 sec. for 2 min. One unit of serum LDH activity is defined as an increase in absorbance of 0.001/min./ml. of serum (*3*). A solution of Evans blue dye or quinidine sulfate yielding an initial absorbance for the reaction mixture of about 0.100 is used as the reference solution. All reactions were performed at 25° using a Beckman DU spectrophotometer equipped with thermospacers connected to a constant-temperature water bath; cuvette compartment temperature varied less than 0.2°.

## Results

The optimum substrate, buffer concentration, and *p*H for the "forward" serum LDH assay were determined using both normal and abnormal sera. Maximum activities for all sera were obtained with a

*Bronwill Constant Temperature Circulator, Will. Corp., Rochester, N. Y.

concentration of 77.5mM DL-lactic acid, 5.3mM of DPN, and 0.05M of sodium pyrophosphate buffer at *p*H 8.8. Other buffers, i.e., 2-amino 2-methyl 1-propanol, or Tris(hydroxymethyl)aminomethane, did not activate the reaction.

The effect of temperature on the LDH activity of two normal and two abnormal sera was determined at 25°, 32°, and 39°. When the log of the activity was plotted against the reciprocal of the absolute temperature, straight lines with identical slopes were obtained. The temperature coefficients calculated from these activities were as follows:*

| *Temperature of reaction (°)* | *Coefficient* |
|---|---|
| 20 | 1.45 |
| 21 | 1.35 |
| 22 | 1.24 |
| 23 | 1.15 |
| 24 | 1.07 |
| 25 | 1.00 |
| 26 | 0.92 |
| 27 | 0.85 |
| 28 | 0.80 |
| 29 | 0.74 |
| 30 | 0.69 |
| 31 | 0.64 |
| 32 | 0.59 |
| 33 | 0.55 |
| 34 | 0.51 |
| 35 | 0.47 |
| 36 | 0.44 |
| 37 | 0.41 |
| 38 | 0.38 |
| 39 | 0.35 |
| 40 | 0.33 |

The reproducibility of this method using frozen premixed reagents was determined by assaying the activity of frozen aliquots of serum over a 12-day period; the mean value was 101 ± 1.35 U., with a coefficient of variation of 1.3%.

The reaction rates were linear in all instances in which the absorbance changed by less than 0.100/min. (Fig. 1, Lactate⟶pyruvate); at higher activities less serum must be used to obtain linear rates. Within the optimal range, activity was directly proportional to the amount of serum assayed, and the calibration curves regressed through zero, con-

*Methods for maintaining constant cuvette temperature are available for the Coenzometer (Macalaster Scientific Corp., Cambridge, Mass.), the Beckman DU, and the Bausch and Lomb Spectronic "20-340" (Rotocell, A. H. Thomas Co., Philadelphia, Pa.) spectrophotometers.

firming the absence of a blank (Fig. 2, Lactate⟶pyruvate). Comparable results were obtained with markedly lipemic or icteric sera.

The serum LDH activity of 70 healthy adults determined by this method ranged from 41 to 99 U. The mean ± 1 standard deviation was 70 ± 14 U. at 25°, and hence, the upper limit of normal was 98 U.

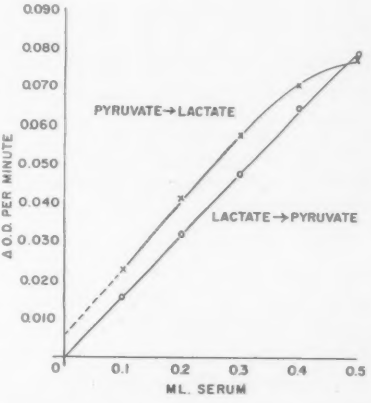



**Fig. 2.** Activity of serum LDH, shown as function of volume of serum assayed according to Henry's method (*5*), is not proportional to volume of serum (upper line, pyruvate to lactate), and significant "blank" is present. Reaction mixture contained 0.05M potassium phosphate buffer, *p*H 7.4; 0.04mM of β-DPNH, and 0.6mM of pyruvic acid per liter. Final volume was 3 ml. per assay. Activity is proportional to amount of serum assayed using present method, and no blank is present (lower line, lactate to pyruvate). Reaction mixture contained 0.05M sodium pyrophosphate buffer, *p*H 8.8; 77.5mM of lactic acid and 5.25mM of DPN per liter. Final volume was 3 ml. per assay. Same serum was employed for both assays.

The spectrophotometric assay (*5*) employing the "backward" reaction (pyruvate + DPNH⟶lactate + DPN) was evaluated in a similar manner. The reaction rates fell off during the 6-min. recording period when the change in absorbance was greater than 0.060/min. (Fig. 1). Moreover, activity was not proportional to the amount of enzyme added to the reaction mixture (Fig. 2). The stability of the frozen reagents for the "backward" reaction was determined over a 10-day period by assaying frozen aliquots of a serum of known activity; during this period the initial activity fell progressively from 340 U./ml. to 270 U./ml. When fresh reagents were substituted for the frozen ones, however, no such change in activity occurred. Thus there was a significant deterioration of the frozen reagents.

The colorimetric method employing 2,4-dinitrophenylhydrazine (*6*) measures the concentration of pyruvic dinitrophenylhydrazone (at 464 mμ) after 30 min. of reaction. The serum LDH activity is then derived from a calibration curve that relates the absorbance of decreasing concentrations of pyruvic dinitrophenylhydrazone to spectrophotometric LDH activities. This method was compared with the serum LDH activity determined by the "forward" reaction (Table 1). Two

**Table 1.** COMPARISON OF "FORWARD" SPECTROPHOTOMETRIC METHOD WITH DINITROPHENYLHYDRAZINE COLORIMETRIC METHOD FOR ASSAYING SERUM LDH ACTIVITY

| *Serum No.* | *Spectrophotometric assay (U.)* | *Colorimetric assay* (U.)* | |
|---|---|---|---|
| | | *Reagent curve A* | *Reagent curve B* |
| 1 | 88 | 47 | 27 |
| 2 | 91 | 59 | 39 |
| 3 | 108 | 53 | 34 |
| 4 | 117 | 73 | 54 |
| 5 | 149 | 98 | 80 |
| 6 | 154 | 87 | 67 |

*In order to compare these data with the "forward" spectrophotometric assay, the units have been converted from the units of the "backward" reaction to those of the "forward" reaction. Thus the upper limit of normal of the "backward" assay, e.g., 350 U., has been set equal to the upper limit of normal of the "forward" assay, e.g., 100 U. All other values are proportional to this ratio.

colorimetric calibration curves were prepared separately from the same reagents: Whereas these two curves appeared to differ from each other very slightly, the serum LDH activities derived from them differed from each other by 19–42%. Moreover, the accuracy of the colorimetric method as compared to that of the "forward" spectrophotometric assay is extremely poor (Table 1). The LDH activities of sera No. 3–6 denoted pathologic increases when assayed spectrophotometrically, but the colorimetric procedure suggested that they were "normal."

## Discussion

The assay methods currently employed for the measurement of serum LDH activity have been evaluated in terms of their simplicity, repeatability, and accuracy. The spectrophotometric assay measuring the reduction of DPN in the presence of lactate offers distinct advantages over the other two methods studied. In comparison to the assay measuring DPNH oxidation in the presence of pyruvate, the "forward" assay has no blank activity and does not require a preincubation period for the consumption of serum substrates by other DPN-dependent serum enzymes, and thus the optimal concentration of DPN is always maintained (*8*). With the "forward" assay, reaction rates are linear over a wide range of activities, and activity is directly proportional to the amount of serum present, precluding frequent dilutions of the serum. Moreover, the "forward" reaction mixture is stable for over 6 months when stored frozen, while the reagents for the

"backward" reaction deteriorate rapidly. Furthermore, DPNH when stored deteriorates spontaneously to form an inhibitor of the LDH reaction (*9*). Finally, the normal range of activity obtained with the present method fits a Gaussian distribution rather than the skewed distribution obtained with the "backward" reaction (*5*). All these factors render the "forward" assay method simpler, more precise, and hence capable of separating normal from abnormal activities with greater certainty than the "backward" assay method.*

On the basis of the above evaluation of the "forward" spectrophotometric assay, we have employed it as a standard of comparison for the colorimetric dinitrophenylhydrazine method. From the data shown in Table 1 the unsuitability of this method is quite evident. This is not unexpected since large variations in activities are shown to result from minor variations in the reagent calibration curve, and since inadequate concentrations of substrate and coenzyme are used in the reaction mixture, activities are not proportional to the concentration of serum enzyme.

Methodologic excellence is essential in any diagnostic procedure, especially in the determination of serum LDH activity, where potentially life-saving decisions may depend on this measurement. Serum LDH activities are invariably elevated during the 10–12 days following an acute myocardial infarction (*3*) or an acute pulmonary embolism (*11*). False-negative results have not occurred in any case proved by autopsy, or in any patient with acute myocardial infarction or acute pulmonary embolism diagnosed by established clinical criteria (*1, 12*). An elevated serum LDH activity may therefore be considered to be a necessary confirmatory criterion for establishing the diagnosis of acute myocardial infarction or acute pulmonary embolism. As such, a normal serum LDH activity can be employed to exclude the recent occurrence of these diseases (*1, 12*).

The use of serum LDH activity in the differentiation of severe angina pectoris from acute myocardial infarction is a special and important diagnostic application of this method, since a value which is erroneously low can lead to the exclusion of an acute myocardial infarction with obvious dangerous consequences.

The finding of an abnormal serum LDH activity warrants a meticulous clinical and laboratory study to determine its cause, since a per-

*The "Coenzometer" (Macalaster Scientific Corp., Cambridge, Mass.) an inexpensive and precise ultraviolet photometer (*10*) with a constant temperature cuvette chamber, has been evaluated for its suitability in the measurement of serum LDH activities. Twelve replicate serum LDH assays performed with this instrument had a coefficient of variation of 3.1%.

sistent elevation of activity may represent the single objective finding in patients suffering from serious and potentially fatal diseases, i.e., liver disease, lymphoma, leukemia, carcinoma, pancreatitis, or "collagen" diseases (*1*). Persistent elevations of serum LDH activity have not been observed in normal persons.

These considerations make it mandatory that normal LDH activities be differentiated clearly from abnormal activities. Optimum substrate concentrations, careful cuvette temperature control, and the absence of blank activities impart to the "forward" spectrophotometric assay described here an over-all precision and simplicity commensurate with its diagnostic significance.

## Addendum

Measurement of urinary LDH activity appears to be an accurate and sensitive screening test for carcinomas of bladder and kidney (*13*). Optimum assay conditions are identical to those described above: the final volume of the reagent solution is 200 ml.; 2.0 ml. are stored at −20°. Urinary LDH activity is measured promptly after collection of timed, clean-voided, overnight 8-hr. urine specimens; no preservative is used. Urine volume is determined and an aliquot centrifuged at 2000 rpm for 5 min. The sediment is examined microscopically, and protein and sugar are determined in the supernatant.

All human urine contains inhibitors of LDH, which are reproducibly removed by dialysis (*14*). Centrifuged urine, 5 ml., is transferred to a cellulose casing (Visking Co., Chicago, Ill. with a ¼-in. diameter, previously washed thoroughly in 3 changes of boiling distilled water, stored in distilled water and knotted at one end. This is then knotted above the urine, weighed, placed in a beaker and dialyzed against running cold tap water for 80 min. It is then reweighed and centrifuged if precipitate is present. Dialyzed urine, 1 ml., is mixed gently with 2 ml. of buffered substrate, and the absorbance is recorded every 2 min. for 10 min. at 340 mμ.

$$\text{Activity} = \text{U/ml.} \times \text{8-hour volume in ml.} \times \frac{\text{weight after dialysis}}{\text{weight before dialysis}}$$

The urinary LDH activities of 47 healthy adults (24 males and 23 females) had a mean ± 1 standard deviation of 1300 ± 375 U./8-hour volume at 25°; hence the upper limit of normal is 2050 U.

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# Automation of Protein-Bound Iodine Determinations

*Clarence O. Stevens* and Nicholas G. Levandoski*

**Portions of the Barker alkali-incineration technic for determining protein-bound iodine have been automated using an automatic analysis system. The manifold and analytic procedure are described in detail. Data are presented to show that the automated procedure compares favorably in accuracy with the manual procedure.**

RECENT YEARS have seen increased clinical use of serum protein-bound iodine values as an aid in assessing thyroid function. Consequently, this and numerous other clinical laboratories have been overburdened with requests for performance of a determination sufficiently complex as to require the devoted attention of a skilled analyst. In order to conserve the time involved for such valuable technical personnel, it was decided to automate certain portions of the Barker alkali-incineration technic (*1, 2*).

## Reagents and Materials

The reagents listed below are prepared according to directions given by Barker *et al.* (*1*), with slight modifications that are described. Demineralized water, containing less than 1 ppm impurities, is used in preparing all solutions.

*Zinc sulfate, 10%* w/v.

From Fitzsimons General Hospital, Denver 30, Colo.

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This material has been reviewed by the Office of the Surgeon General, Department of the Army, and there is no objection to its presentation and/or publication. This review does not imply any indorsement of the opinions advanced or any recommendation of such products as may be named.

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*Sodium hydroxide, 0.5 N*
*Sulfuric acid, 7.0 N*
*Hydrochloric acid, 2.0 N*
*Sodium arsenite, 0.1 N*

*Acid mixture* Combine the following in a 1 L. polyethylene bottle and mix thoroughly: sulfuric acid, 7.0 *N*, 286 ml.; hydrochloric acid, 2.0 *N*, 286 ml.; demineralized water, 428 ml.

*Sodium carbonate, 4.0 N*

*Potassium iodide, stock standard, 200 μg. iodide per milliliter* Dissolve 261.6 mg. dry potassium iodide in water and dilute to a final volume of 1 L., using a volumetric flask.

*Working potassium iodide standards* Prepare working standards fresh before use of 0.005, 0.010, 0.020, 0.030, 0.040, 0.050, 0.075, and 0.100 μg./ml. from an 0.200-μg./ml. diluted stock solution.

*Ceric ammonium sulfate, 0.008 N* Dissolve 4.635 gm. anhydrous ceric ammonium sulfate* in 500 ml. water to which has been added 92 ml. 7.0 *N* sulfuric acid. When the solution is clear dilute to a final volume of 1 L. in a volumetric flask. Against water as a blank, the percentage transmittance of this solution should be 14.0 at 420 mμ. If the transmittance of this solution does not fall between 13.0 and 15.0%, adjust the strength of the solution by adding water or more ceric ammonium sulfate.

*Pipetter, Cornwall continuous, syringe type, B-D*†

*Automatic pipet with volume selector, 2 ml.*‡ This pipet is used for delivery of 10% zinc sulfate, 0.5 *N* sodium hydroxide, and 4.0 *N* sodium carbonate.

*Technicon AutoAnalyzer*§ Necessary modules include a recorder, colorimeter, proportioning pump, sampling mechanism and two 38° heating baths.

## Analytical Procedure

The blood sample is centrifuged and the serum drawn off. Duplicate aliquots of 1 ml. are added to 7 ml. demineralized water in 15 × 125-mm. Pyrex test tubes. Duplicate reagent blank tubes are prepared containing 8 ml. demineralized water. Two glass beads are added to each tube. Following this, 1 ml. 10% zinc sulfate is added to each tube

*A satisfactory grade of this chemical can be purchased as reagent grade from G. Frederick Smith Chemical Co., Columbus, Ohio.

†Becton, Dickinson & Company, Rutherford, N. J.

‡Available from Scientific Products, Division of American Hospital Supply Corp., North Kansas City 16, Mo.

§Technicon Instruments Corp., Chauncey, N. Y.

and the contents mixed by gently tapping the side of the tube until the protein precipitate is evenly distributed. Then 1 ml. of 0.5 *N* sodium hydroxide is added to each tube and the contents mixed again.

The tubes are centrifuged for 8 min. at 3000 rpm in head 250 of an International Size 2 centrifuge and the clear supernatant fluid discarded. Then 7 ml. demineralized water is added to each tube and the protein precipitate broken up by tapping the side of the tube. Tubes are again centrifuged for 8 min. at 3000 rpm and the supernatant discarded. Washing with 7 ml. water is repeated twice. To the resulting precipitate in each tube is added 1 ml. 4.0 *N* sodium carbonate. The precipitate is evenly suspended by tapping the side of the tube.

The tubes are then placed in drying racks constructed in such a manner that the tubes lie on their sides with the open ends elevated about ½ in. The racks are placed in a drying oven maintained at 92–94° at an atmospheric pressure of 630 mm. for 20–24 hr., until the protein-sodium carbonate mixture is thoroughly dry. The racks of dry tubes are transferred to a muffle furnace preheated to 95° and the temperature is raised to 600 ± 25° and kept there for 24 hr. The furnace is then turned off and the racks left in the furnace overnight to cool slowly. The ash is treated according to directions for the manual or automatic procedure given below.

### Manual Procedure

To each ashed tube is added 8 ml. acid mixture. The first 1.5 ml. is added dropwise, washing down the upper walls of the tube. Each tube is agitated until effervescence ceases. The tubes are centrifuged for 20 min. at 3000 rpm and the clear supernatant acid mixture transferred to labeled 15 × 125-mm. Pyrex test tubes. No carbon particles should be carried over in the supernatant. For each blank and unknown tube, two 19 × 150-mm. cuvets are labeled A and B. Three milliliters of the acid solution is transferred from each tube to each A and each B cuvet representing the sample. Then, 1 ml. potassium iodide, 0.04 μg./ml., is added to each B cuvet and 1 ml. demineralized water is added to each A cuvet. To each cuvet is added 1 ml. 0.1 *N* sodium arsenite. The contents of each cuvet is mixed by tapping the sides. The cuvets are placed in a 38° water bath together with a cuvet containing 8 ml. water to be used as a blank to set the Coleman Jr. spectrophotometer at 100% transmission. Also, a test tube containing 0.008 *N* ceric ammonium sulfate is placed in the water bath. After 10 min. of temperature equilibration of the tubes, the spectrophotometer is adjusted to 100%

transmission at 420 mμ using the water blank. Using a stopwatch to time the intervals, 1 ml. of the equilibrated 0.008 *N* ceric ammonium sulfate is added to each cuvet at exactly 30-sec. intervals. Immediately after addition of the ceric ammonium sulfate, the contents of each cuvet are mixed by sharply tapping the side of the cuvet. Thirty seconds after ceric ammonium sulfate has been added to the last cuvet, a percentage transmission reading is taken on the first cuvet. Limitations in time require that no more than eight cuvets be treated with ceric ammonium sulfate at one time. Each 30 sec., a percentage transmission reading is taken on the next cuvet in line. When a reading has been obtained on each cuvet, the readings are started again in the same order every 30 sec. Readings are continued on each cuvet until there are at least three percentage transmission readings greater than 25 or until the reading has become greater than 65 for the cuvet.

A graph is made of the percentage transmission readings versus incubation time in minutes. The best straight line is drawn between the points representing readings between 25 and 60% transmission. When both the lines for the A and the B cuvet readings for any one sample have been drawn, some convenient horizontal line is drawn intersecting both lines A and B. The horizontal line chosen should be such that the difference in time between the intersection with line A ($T_1$) and the intersection with line B ($T_2$) is at least 10 min. The values of $T_1$ and $T_2$ are substituted in the following equation:

$$\mu\text{g./100 ml. iodide} = \frac{0.04\ T_2}{T_2 - T_1} \times \frac{8}{3} \times 100$$

The average value for the blank is subtracted from the average value for each unknown to give the protein-bound iodine value in micrograms per 100 ml. serum.

### Automatic Method

To each ashing tube is added 5 ml. acid mixture. The first 1.5 ml. is added dropwise, washing down the upper walls of the tube. Each tube is agitated until effervescence ceases. The tubes are centrifuged for 20 min. at 3000 rpm and the clear supernatant acid mixture transferred to labeled 15 × 125-mm. Pyrex test tubes. No carbon particles should be carried over in the supernatant. For each blank and unknown tube, two 12-ml. conical centrifuge tubes are labeled A and B. To Tube A is added 0.2 ml. water while 0.2 ml. potassium iodide, 0.2 μg. iodide per milliliter, is added to Tube B. From each ashing tube, 1.8 ml. of the

acid solution is transferred to each of a pair of A and B tubes. The contents of each tube are mixed by tapping the side and are then transferred to a sampling cup on the AutoAnalyzer as described below.

The AutoAnalyzer is assembled as indicated in Fig. 1. Detailed descriptions of the operation of this instrument have been published by the Technicon Instruments Corporation (*3*). The plastic tubing manifold is replaced with a new one every 2 months. Demineralized water is aspirated by the proportioning pump through all reagent lines and the recorder adjusted to 100% transmission using a 1.0-cm. flow cell and 420-m$\mu$ filters in the colorimeter. When the instrument has stabilized at 100% transmission, reagent lines are placed in the indicated bottles. After 10 min., the sampling mechanism is started so that it dips into the first plastic cup. Working standards and samples are arranged on the sample plate in the following manner. Four cups of acid mixture precede the first working standard cup. Two cups of acid mixture are placed between each working standard cup. After the last working standard cup come five cups of acid mixture. Specimen portion A is placed in the next cup and is followed by one cup of acid mix-

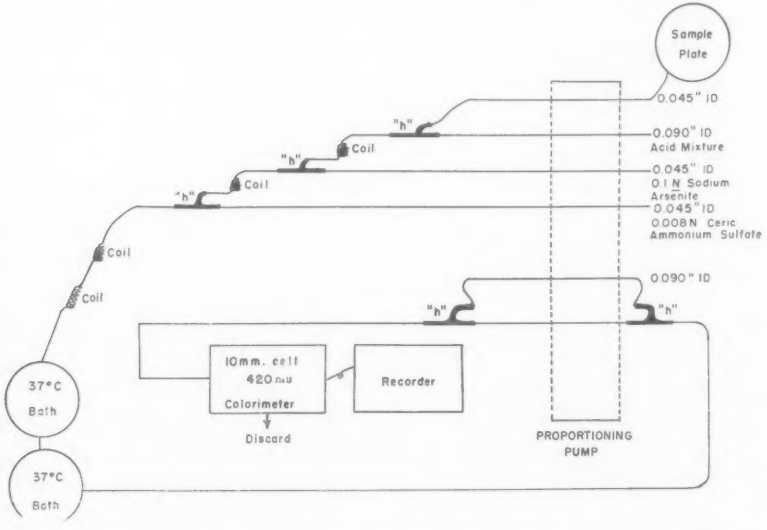


**Fig. 1.** Automatic system for protein-bound iodine determinations. Reagents enter through the lines designated. All components are standard accessories to the Technicon AutoAnalyzer. Mixing coils used are described as "single length."

ture, specimen portion B, and two cups of acid mixture. The next specimen follows in the same manner. As many as 20 specimens may be run in sequence. At the beginning and end of a series of determinations, a complete set of working standards is run.

The percentage transmission at the top of each peak is recorded and a standard curve constructed from the values obtained from the various working standard concentrations. From the standard curve, the concentration of iodide present in specimen A and B portions is determined. The concentration of iodide present in the ashed blank and serum specimens is calculated as follows.

$$\mu g./100 \text{ ml. iodide} = \frac{x\ (2.0)\ (5.0)\ (100)\ (0.02)}{(y - x)\ 1.8} = \frac{11.1\ x}{y - x}$$

where $x$ is micrograms iodide per milliliter in specimen portion A and $y$ is micrograms iodide per milliliter in specimen portion B.

The average blank value is subtracted from each average specimen value to obtain micrograms protein-bound iodine per 100 ml. serum.

## Results and Discussion

A typical record of standards is depicted in Fig. 2. Iodide concentration determines the extent of reduction of ceric ion during incuba-

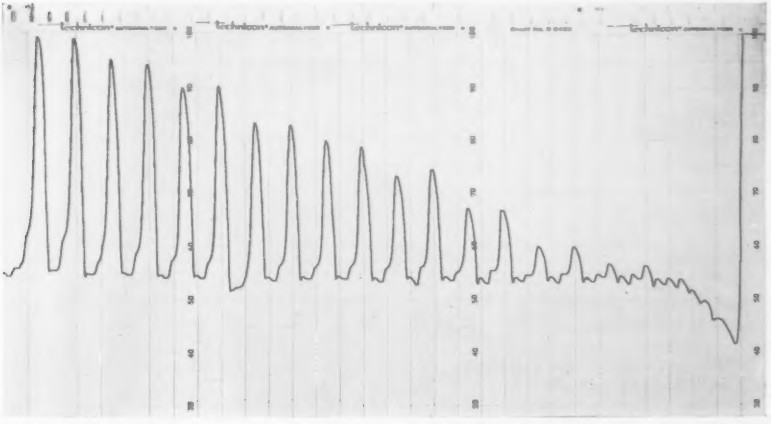

**Fig. 2.** AutoAnalyzer record of iodide standards. Each pair of peaks from left to right represents duplicate potassium iodide working standard cups at each of the following iodide concentrations, in micrograms per milliliter: 0.2, 0.1, 0.075, 0.05, 0.04, 0.03, 0.02, 0.01, and 0.005.

tion at 38°. The extent of ceric ion reduction is reflected by decolorization or an increase in percentage transmission at 420 mμ. Such an inverse colorimetric technic makes it desirable to run working standard concentrations in duplicate. Generally, there is good agreement be-

**Table 1.** Comparison of Results from Automatic and Manual Methods for the Determination of Serum Protein-Bound Iodine

| | *Protein-bound iodine (μg./100 ml.)* | | | |
|---|---|---|---|---|
| | *Manual method* | | *Automatic method* | |
| *Specimen* | *Duplicate values* | *Av.* | *Duplicate values* | *Av.* |
| Patient 1 | 5.96 | 6.00 | 6.00 | 6.01 |
| | 6.04 | | 6.02 | |
| Patient 2 | 7.06 | 6.94 | 6.81 | 6.64 |
| | 6.82 | | 6.47 | |
| Patient 3 | 2.37 | 2.29 | 2.31 | 2.43 |
| | 2.21 | | 2.56 | |
| Patient 4 | 6.29 | 6.39 | 6.40 | 6.53 |
| | 6.50 | | 6.66 | |
| Patient 5 | 7.12 | 7.24 | 7.42 | 7.55 |
| | 7.35 | | 7.68 | |
| Patient 6 | 2.62 | 2.80 | 2.76 | 2.72 |
| | 2.98 | | 2.68 | |
| Patient 7 | 5.13 | 5.26 | 5.21 | 5.14 |
| | 5.48 | | 5.07 | |
| Patient 8 | 4.43 | 4.58 | 4.48 | 4.44 |
| | 4.74 | | 4.40 | |
| Patient 9 | 6.79 | 6.78 | 6.52 | 6.42 |
| | 6.77 | | 6.29 | |
| Patient 10 | 12.10 | 12.29 | 12.21 | 12.27 |
| | 12.48 | | 12.33 | |
| Chemtrol* | 6.36 | 6.22 | 6.67 | 6.44 |
| | 6.07 | | 6.20 | |
| Versatrol† | 5.94 | 5.63 | 5.85 | 5.65 |
| | 5.31 | | 5.45 | |
| Versatrol-A‡ | 3.42 | 3.22 | 3.44 | 3.24 |
| | 3.01 | | 3.05 | |
| Hyland abnormal clinical chemistry control serum§ | 10.33 | 10.55 | 10.50 | 10.46 |
| | 10.77 | | 10.42 | |

*Lot No. 9250, Clinton Laboratories, Los Angeles 36, Calif. Value given for lot is 6.3, with an acceptable range of 5.9–6.5.

†Lot No. VO299040, General Diagnostics Division, Warner-Chilcott Division, Morris Plains, N. J. Value given for lot is 5.7.

‡Lot No. VA35319, General Diagnostics Division, Warner-Chilcott Division. Value given for lot is 3.1.

§Lot No. 368R1, Hyland Laboratories, Los Angeles, Calif. Value given for lot is 10.3, with an acceptable range of 9.7–10.9.

tween duplicates. With two cups of acid mixture between working standard cups, no significant cross-contamination is apparent in the working standards employed.

In constructing the standard curve, the average percentage transmission value at the top of duplicate peaks is plotted against iodide concentration. The most linear and, therefore, reliable portion of this curve lies between 0.005 and 0.05 $\mu$g. iodide per milliliter, corresponding to 2.74 and 27.4 $\mu$g. iodide per 100 ml., respectively. Most specimens from clinical sources at this hospital fall within this range.

In Table 1, results of a typical series of determinations are presented. Duplicate values from either method agree closely with each other. Further, there is close agreement between the average results for the manual and automatic methods, the widest variation being about 6% for Patient 3. Though the highest value listed is 12.29, for Patient 10, satisfactory results have been obtained at rare higher levels up to 50 $\mu$g. protein-bound iodine per 100 ml. Above 50 $\mu$g. protein-bound iodine per 100 ml., the specimen must be set up again using a smaller amount of serum for precipitation and ashing.

The automatic method is as satisfactory as the manual method, and results in a great saving in time and glassware.

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1. Barker, S. B., Humphrey, M. J., and Soley, J. H., *J. Clin. Invest.* **30**, 55 (1951).
2. Thompson, H. L., Klingerman, M. R., and Truemper, J., *J. Lab. Clin. Med.* **47**, 145 (1956).
3. *Technicon AutoAnalyzer Methodology File*, copyright 1958 by Technicon Instruments Corp., Chauncey, N. Y.

# Protein-Bound Iodine, Total Iodine, and Butanol-extractable Iodine by Partial Automation

*Joseph Benotti and Norbert Benotti*

**The determination of iodine compounds in serum is simplified by using an automatic system to record the decolorization of the ceric sulfate solution. A method for modifying existing technics for PBI and BEI determinations, prior to this step, is described. Good agreement between the automated and manual technics was achieved.**

Over the past several years, the measurement of protein-bound iodine (PBI) and butanol-extractable iodine (BEI) has become quite popular in evaluating thyroid function. So great has the demand been for these determinations that some laboratories are performing hundreds of the assays per day. In view of these facts, it becomes obvious that automation should be utilized as much as possible to offset the shortage of trained personnel.

Since all of these procedures involve the determination of organic iodine bound to protein, some type of digestion is necessary to convert the iodine into its inorganic form, which may be done by either wet ashing or dry ashing. Wet ashing involves digestion with a strong acid, while the dry-ash technic makes use of a muffle furnace and an alkali medium. Attempts are being made to carry out digestion by automation, but without too much success. Consequently, we have not resorted to automation for the purposes of digestion.

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From the Boston Medical Laboratory, Boston 15, Mass.

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## Materials and Methods

### Components of Automatic Analyzer*

The components of the automatic analyzer we use are as follows:

1. Sampler.
2. Pump.
3. Constant-temperature water bath controllable between 28° and 37° with 80-ft. glass coil.
4. Colorimeter.
5. Recorder.

The flow diagram used for reading iodine determinations is shown in Fig. 1.

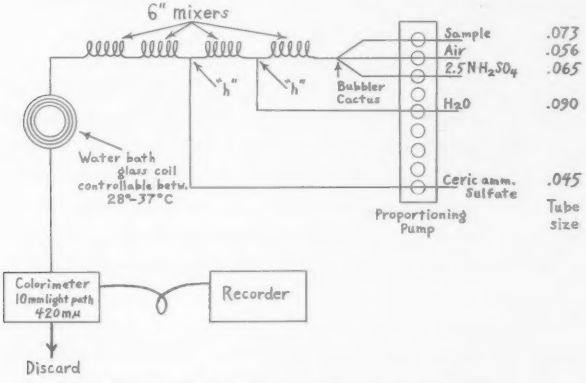


**Fig. 1.** Flow diagram for automatic analyzer.

### Reagents

*Chloric acid* To a 3-L. Florence flask are added 500 gm. of potassium chlorate (reagent grade) and 910 ml. of distilled water. The potassium chlorate is dissolved by heating. With constant stirring, 375 ml. of 70% perchloric acid are added from a separatory funnel at a rate of approximately 15 ml./min. After cooling, the acid is placed in a freezer overnight at a temperature of −23°. The next morning the chloric acid is filtered with the aid of suction through a Buchner funnel using Whatman No. 1 filter paper. This preparation yields approximately 1 L. of 28% chloric acid.

*Arsenious acid* To a 2-L. Florence flask are added 10 gm. of

*Auto-Analyzer, Technicon Co., Chauncey, New York.

arsenic trioxide, 50 gm. of sodium chloride, 400 ml. of 5N $H_2SO_4$, and approximately 1 L. of distilled water. The contents are heated until solution is complete. After cooling, the volume is made up to 2 L. with distilled water.

*5N Sulfuric acid* Approximately 1 L. of distilled water is placed in a 2-L. Florence flask to which is slowly added 280 ml. of conc. $H_2SO_4$. After cooling, the volume is made up to 2 L. with distilled water.

*Ceric ammonium sulfate** Ten grams of ceric ammonium sulfate is dissolved in a liter of 2.5N $H_2SO_4$.

*"Special screening" ceric ammonium sulfate* Sixteen grams of ceric ammonium sulfate is dissolved in a liter of 2.5N $H_2SO_4$.

*Alkali wash solution for BEI's* Each liter of water has dissolved in it 160 gm. sodium hydroxide and 50 gm. sodium carbonate.

## Procedure

The method of digestion we have adopted is essentially that of Zak *et al.* (*1*) with some slight modifications. This involves wet ashing with chloric acid. The reasons we have resorted to this method are several: (1) Material to be analyzed stays in the same tube from the beginning of the procedure to the end. (2) Multiple analyses may be performed at one time. (3) No distillation of iodine is necessary after digestion. (4) Time of digestion is only 2 hr.

### PBI Precipitation

To 0.5 ml. of serum in a 40-ml., round-bottom centrifuge tube (Pyrex No. 8260), add rapidly 10 ml. of 5% trichloracetic acid by the blowing from a wide-bore serological pipet. The force of the stream will disperse the precipitated protein in fine particles, resulting in better extraction of extraneous inorganic iodide from the protein. For large numbers of specimens, we use an automatic pipet (Brewer automatic pipetting machine) which delivers 10 ml. of 5% trichloracetic acid. The precipitate is allowed to stand for 20 min. to coagulate the proteins; then the tubes are centrifuged for 10 min. The supernatant fluid containing inorganic iodine is decanted and discarded. The packed protein is resuspended by blowing 10 ml. of 5% trichloracetic acid in a fast stream to disperse the packed protein, recentrifuged, and the supernatant fluid discarded as before. The protein is then ready for digestion.

*G. Frederick Smith Co., Columbus, Ohio.

### Total Iodine

The digestive acid is added directly to 0.5 ml. serum, and the digestion is carried out as described under *Digestion.*

### Butanol-extractable Iodine

There are times when a patient has had extraneous iodide for various reasons over a long period. In such cases, the PBI may be falsely elevated. Furthermore, it may be of clinical advantage to evaluate the true thyroxin level in the blood. This is best carried out by performing the BEI determination. The procedure used is a modification of that of Man *et al.* (*2*).

To 0.5 ml. of serum in a 40-ml. g. s. centrifuge tube (Kimax 45197), is added 0.3 ml. of 10% $H_2SO_4$ and 4 ml. of butanol. The tube is shaken horizontally in a shaking machine for 5 min. After the mixture is centrifuged, the supernatant liquid is decanted into another 40-ml. g. s. centrifuge tube. Again 4 ml. butanol is added to the original tube containing the precipitated protein. This is stirred with an applicator stick, shaken again for 5 min., and recentrifuged. The resulting supernatant liquid is added to the original extract. To the combined butanol extracts 8 ml. of alkali wash solution (5% $Na_2CO_3$ in 4N NaOH) is added, and the whole shaken again on the shaking machine for 5 min. and then centrifuged. The alkali bottom layer is drawn off by means of a capillary tube attached to a vacuum pump. After 8 ml. of alkali wash solution is added to the butanol fraction the whole is shaken again and centrifuged, and the bottom layer drawn off as above. The resultant butanol extract is poured off into a 40-ml. centrifuge tube which is used for iodine digestion. Six drops of 6N HCl are added to the butanol fraction. The butanol is finally evaporated by placing the tubes in an aluminum-block heater* set at 120°. The evaporation is enhanced by placing a glass tube in each digestion tube and connecting directly to a suction manifold as shown in Fig. 2. This in turn is permanently connected by a duct directly to the main suction duct of the fume hood. By this technic, it takes less than 20 min. to evaporate 8 ml. of butanol to complete dryness. From this point on, the BEI's are treated as regular iodine determinations; that is, chloric acid is added, and they are digested and read as described below.

### Digestion

To the material to be analyzed, containing less than 0.1 $\mu$g. of iodine, is added 3 ml. of chloric acid. To make the chloric acid a more powerful

*Constantemp, Roeco Manufacturing Service, South San Gabriel, Calif.

oxidizing agent, 1 mg. of sodium chromate is added to each tube. This is best done by adding 1 ml. of 33.3% sodium chromate $4H_2O$ to 1 L. of chloric acid. This latter solution contains 1 mg. of sodium chromate per 3 cc. of chloric acid.

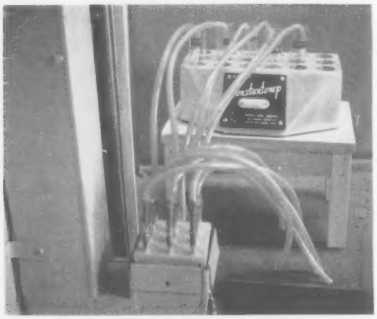

**Fig. 2.** Butanol evaporation assembly.

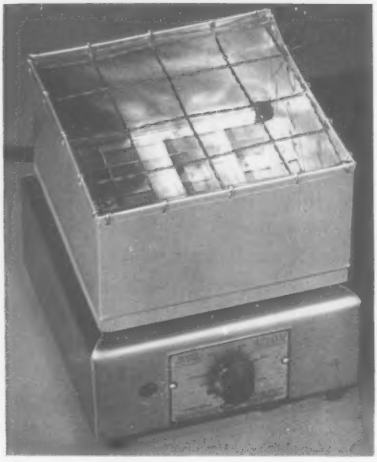

**Fig. 3.** Heater digestion assembly.

The digestion is accomplished in a heater* equipped with a special superstructure† designed by Benotti and Benotti as shown in Fig. 3. One pound of fine sand, reagent grade (Merck) is placed in the heater. The temperature is set between 105° and 110°. The tubes containing

*Temco, Thermolyne Corp., Dubuque, Iowa.
†E. F. Mahady Co., Cambridge, Mass.

the protein and chloric acid are set in the sand by gentle pressure and left for one hour without further observation. At the end of this time, the tubes have to be watched rather closely for color changes such as orange-yellow to light green to colorless. This change is due to the reduction of chromic to chromous ion. It is important that this be prevented (in order to avoid loss of iodine) by adding a drop or two of chloric acid. This restores immediately the original orange-yellow color. After another hour of close observation and occasional addition of chloric acid drop by drop, the digestion should be complete. At removal of the tubes from the heater and upon consequent lowering of their temperature, red crystals of chromium trioxide appear on the glass. If such red crystals do not appear, the tubes must be put back in the heater until red crystals appear.

### Colorimetry of Iodine Determinations

The colorimetry of the iodine determination is essentially the same as that performed by manual operation except that it has been automated. The mechanism of the catalytic reaction between arsenic and cerium has been adequately brought out by Sandell and Kolthoff (*3*). After digestion is complete by the technic described above, about 0.5 ml. of solution remains in the tubes plus some red crystals of chromium trioxide. To this is added 2.1 ml. of arsenious acid reagent by means of a Brewer automatic pipetting machine. This is mixed and 2 ml. poured into the sample cup of the AutoAnalyzer. The portion remaining in the original digestion tube is used for screening for grossly contaminated specimens. This is performed by adding one drop of "special screening" ceric ammonium sulfate solution. If the yellow color persists for 1½ min., the iodine value may be read on the instrument. If the color fades within 1½ min., the values are too high to read and the specimen must be removed from the sampler plate in order to prevent contamination of the automatic analyzer. Samples which fade within 1 min. have an iodine concentration considerably above 20 μg./100 ml. and are usually obtained from patients who had received radiopaque media. Those that fade by 1 and 1½ min. are reported as over 20 μg./100 ml.

Since there is room for 16 digestion tubes in each heater, we always run 4 standards and 12 unknown iodine determinations together as a group. The results as recorded on the automatic analyzer are given for one group in Fig. 4. Reading from the bottom up, the first peak represents a blank determination and the next 3 represent standards of 0.02,

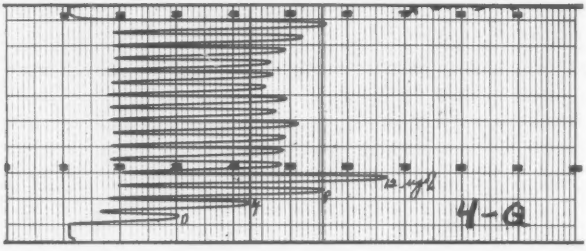


**Fig. 4.** Typical recorder graph tracing for a group of iodine determinations.

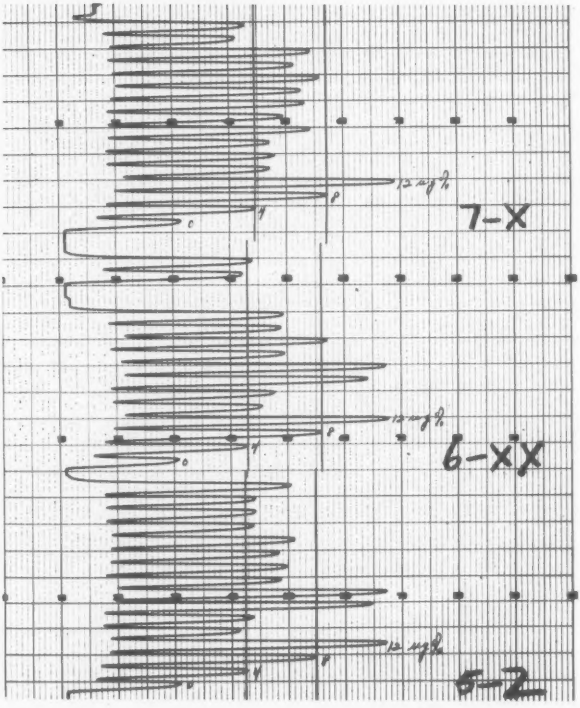


**Fig. 5.** Recorder graph tracing for 3 groups of iodine determinations, showing variation of standards with different heating units.

0.04, and 0.06 μg. of iodine which, when multiplied by the dilution factor of 200, give 4, 8, and 12 μg./100 ml. iodine.

Since the temperature of different heaters may vary by a few degrees, standards should be run with each group. Figure 5 shows how

**Table 1.** Comparison of Manual Colorimetric Technic and Technic by Automatic Analyzer

| *Manual* | | *Automatic System* | |
|---|---|---|---|
| *PBI* | *TI* | *PBI* | *TI* |
| 1.0 | 1.2 | 1.2 | 1.4 |
| 3.2 | 3.8 | 4.0 | 4.0 |
| 3.4 | 3.4 | 3.8 | 4.4 |
| 3.4 | 3.4 | 4.0 | 4.0 |
| 3.4 | 4.6 | 4.2 | 4.2 |
| 3.6 | 4.0 | 3.4 | 4.2 |
| 3.6 | 5.0 | 4.4 | 5.6 |
| 4.0 | 4.2 | 4.4 | 4.4 |
| 4.0 | 4.6 | 4.0 | 4.2 |
| 4.0 | 5.4 | 4.2 | 6.6 |
| 4.4 | 4.6 | 5.0 | 5.2 |
| 4.6 | 4.8 | 4.8 | 4.8 |
| 4.8 | 5.8 | 4.8 | 6.0 |
| 4.8 | 7.8 | 5.0 | 8.4 |
| 5.2 | 6.0 | 5.6 | 7.0 |
| 5.4 | 6.8 | 5.8 | 7.6 |
| 5.4 | 11.4 | 6.0 | 11.2 |
| 5.6 | 6.4 | 6.0 | 7.6 |
| 5.6 | 6.6 | 6.6 | 7.4 |
| 5.8 | 6.2 | 5.8 | 6.2 |
| 5.8 | 6.2 | 5.8 | 6.4 |
| 6.0 | 6.4 | 6.0 | 6.6 |
| 6.2 | 6.4 | 6.2 | 6.8 |
| 6.2 | 7.6 | 6.2 | 7.0 |
| 6.6 | 6.6 | 7.2 | 7.2 |
| 6.6 | 6.6 | 7.6 | 8.0 |
| 6.6 | 6.8 | 7.6 | 8.0 |
| 6.6 | 7.8 | 7.0 | 7.0 |
| 7.2 | 7.6 | 7.0 | 7.6 |
| 7.2 | 8.6 | 7.4 | 8.8 |
| 7.6 | 8.6 | 7.8 | 8.0 |
| 7.8 | 8.2 | 7.2 | 8.0 |
| 8.0 | 8.6 | 8.8 | 9.2 |
| 8.4 | 8.4 | 8.6 | 9.2 |
| 8.6 | 9.6 | 8.8 | 10.0 |
| 8.8 | 9.2 | 9.2 | 9.6 |
| 10.2 | 11.0 | 11.2 | 12.0 |
| 15.2 | 16.0 | 14.8 | 17.2 |

the standards may differ slightly from group to group in various heaters. Here one can see the variation among 3 heating units.

## Discussion

The most tedious and exacting part of the iodine determination is the adding of the several reagents necessary for the proper color development and, finally, the actual spectrophotometric or colorimetric reading. This becomes more obvious when the number of iodine determinations is large. By means of an automatic analyzer system, it has been possible to automate these steps. This in itself is very important because potential human errors have been replaced by mechanical manipulations which do not tire. It is the feeling of the authors that the accuracy of automation as compared to manual reading is greater, particularly when there are many determinations.

As to the reliability of the results obtained by automation, we have run comparative analyses of both methods, as shown in Table 1. It would appear that the manual results are slightly lower than the automated results—0.3 $\mu$g./100 ml. for this small series.

## References

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2. Man, E. B., Kydd, D. M., and Peters, J. P., *J. Clin. Invest.* **30,** 531 (1951).
3. Sandell, E., and Kolthoff, I. M., *Mikrochim. Acta* **1,** 9 (1937).

# Quantitative Range of Nessler's Reaction with Ammonia

*Reid H. Leonard**

**The minimum concentration of mercury required for Nessler's reaction with ammonia approaches 86% of the stoichiometric value. When the mercury concentration is low, nitrogen can be determined in small amounts in the ultraviolet region. The maximum accuracy and working range are shown for 350–600 mμ.**

THE quantitative reaction of Nessler's reagent with ammonia is represented by the following equation from Remy (*1*):

$$2\,(HgI_4)^{=} + NH_3 + 3\,OH^- \longrightarrow (O\begin{matrix}Hg\\Hg\end{matrix}NH_2)\,I + 7\,I^- + 2H_2O$$

or, in balanced form:

$$2\,(HgI_2{\cdot}2KI) + NH_4OH + 3NaOH \longrightarrow OHg_2NH_2I + 3H_2O + 4KI + 3NaI$$

There is considerable variation in ways of preparing Nessler's reagent as well as in the manner of forming the colored ammonobasic mercuric iodide. Two critical values are the ratio of KI to $HgI_2$, i.e., $x$ in $HgI_2{\cdot}x$KI, and the final concentration of mercury. The value $x$ determines the stability of solutions and to some extent the relative sensitivity to $NH_3$. Vanselow (*2*) concluded that $x$ should be 2.10, which is a 5% excess of KI over the theoretical 2 moles. Table 1 shows values of $x$ and the concentration of mercury in several reagent preparations.

Table 2 shows a range of $x$ between 2 and 3. The mercury concentration in the final color solution ranges from 0.002 to 0.02 moles per liter. In view of a 10-fold range, practical consideration would indicate a need for knowledge of the lowest workable concentration.

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*Address: 2544 Escambia Ave., Pensacola, Fla.

Table 1. Composition of Various Nessler's Reagents

| Reagent | x in $HgI_2 \cdot xKI$ | Mercury concentration (M/L.) | |
|---|---|---|---|
| | | Alkaline Hg reagent | Final color solution |
| Fresenius (6) | 2.40 | 0.0479 | 0.0018 |
| A. O. A. C. (7) | 2.91 | 0.1207 | |
| Std. Meth. Cl. Chem. (8) | 2.04 | 0.0754 | 0.0215 |
| Std. Meth. Cl. Chem. (9) | 2.29 | 0.0288 | 0.0086 |
| Std. Meth. Water Anal. (10) | 1.92 | 0.220 | 0.0043 |
| Vanselow (2) | 2.10 | 0.100 | 0.0050 |
| Natelson (11) | 2.10 | 0.0748 | 0.0224 |

## Methods

Chemicals were Baker A.R. grade. Glassware was Class A except for 15 × 120-mm. tubes calibrated in the laboratory for 10-ml. volume (± 0.1 ml.). Absorbance readings were made with a Beckman DB spectrophotometer* in 1-cm. cells.

Mercury solutions were prepared by dissolving the required amount of $HgI_2$ in the KI solution but in a small volume, about 1/100 of the final volume. Then a freshly filtered solution of gum ghatti sufficient for a final concentration of 0.1% was added. The mixture was then made to volume.

Color reactions with ammonia were made in 10-ml. calibrated tubes. In order, ammonia sample, mercury solution, alkali, and water to volume were added and then mixed. This required the use of 2 reagents, a mercury solution and 3N NaOH, in place of the usual mixed Nessler's reagent (3).

### Amount of KI with $HgI_2$

Solutions of the theoretical complex, $HgI_2 \cdot 2KI$, are unstable and precipitate easily, especially when made alkaline. Some excess KI is needed for good stability, but excess KI can lower the ammonia color intensity. The effect of varying KI on a single mercury concentration is shown in Table 2. With a final mercury concentration of 0.0015 M, a maximum color intensity was obtained with $HgI_2 \cdot 2.5KI$.

### Absorption Spectrum

Figure 1 shows the absorbance curves for four concentrations of $HgI_2 \cdot 2.5KI$ in 0.6 N NaOH. In order to read the ammonia color at 350

*Beckman Instruments, Inc., Palo Alto, Calif.

**Table 2.** Effect on Color of Amount of KI in Mercury Solution

| Wavelength (mμ) | Absorbance* | | | | |
|---|---|---|---|---|---|
| | M KI/M $HgI_2$ | | | | |
| | 2.00 | 2.25 | 2.50 | 2.75 | 4.00 |
| 400 | 0.93 | 0.94 | 1.00 | 0.92 | 0.83 |
| 425 | .676 | .684 | .72 | .662 | .624 |
| 450 | .490 | .500 | .522 | .472 | .454 |
| 475 | .352 | .360 | .378 | .342 | .326 |
| 500 | .238 | .244 | .260 | .230 | .222 |
| Total: | 2.686 | 2.728 | 2.880 | 2.626 | 2.456 |

*Composition of color: 3.2 μg. ammonia N/ml., 0.6 N NaOH (4), 0.0015 M mercury.

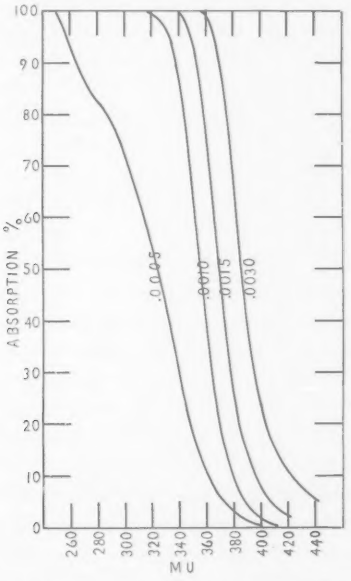


**Fig. 1.** Absorption spectrum of Nessler's reagent.

mμ, it is necessary to keep the mercury concentration to about 0.0005 M; whereas, at 400 mμ about 0.0030 M mercury can be tolerated.

### Ringbom Curves for Ammonobasic Mercuric Iodide

Figure 2 shows the absorbance vs. log of concentration for ammonobasic mercuric iodide at 6 different wavelengths. Figure 2 is the Ring-

bom treatment which was shown by Ayres (*5*) to be useful in establishing accuracy and range of a color system. Figure 2 was prepared by using three different concentrations of $HgI_2 \cdot 2.5KI$, which are noted at the top of the figure.

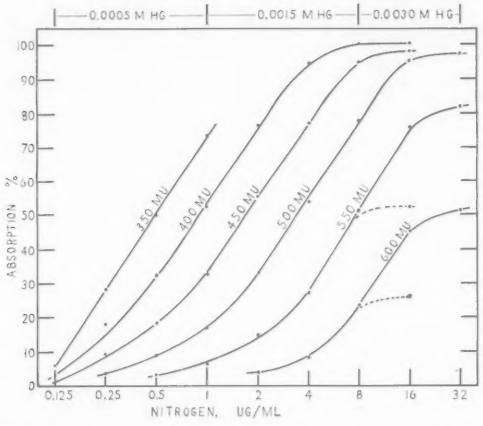


**Fig. 2.** Ringbom curves for ammonobasic mercuric iodide.

The two broken curves on the right show the exhaustion of the available mercury in the 0.0015-M solution with about 9 $\mu$g. of nitrogen. Similar breaks, not shown, were found at about 3 $\mu$g. of nitrogen and, represented the upper level for 0.0005 M mercury and at 20 $\mu$g. of nitrogen for 0.0030 M. In summary, these breaks indicate that 1 $\mu$M of mercury is sufficient for the quantitative measurement of about 0.43 $\mu$M of nitrogen which is 86% of the theoretical value.

The range and accuracy for various wavelengths are shown in Table 3. The slope of the center portion of the Ringbom plot indicates the accuracy with steep slopes preferred. Since little variation was found, there is no preference of one wavelength over another. The relative analysis error (*5*) for 1% photometric error calculates between 3.0% and 3.3% for the DB instrument. Maximum accuracy is at the center inflection point of the Ringbom plot. Calculated minimum mercury concentration is from (1) the upper limit of the range and (2) the extent of reaction found in this article. This value is meant as a guide in formulating reaction mixtures.

Table 3. Accuracy and Range of Nessler's Reaction with Ammonia

| Wavelength (mμ) | Slope index of accuracy (% absorption/cycle) | Ammonia N concentration (μg./ml.) | | Calculated minimum Hg conc. (μM/ml.) |
|---|---|---|---|---|
| | | At maximum accuracy | Range | |
| 350 | 75 | 0.5 | 0.1– 1 | 0.17 |
| 400 | 72 | 1.0 | 0.5– 3 | 0.50 |
| 450 | 73 | 2.0 | 1.0– 5 | 0.83 |
| 500 | 74 | 4 | 2 –10 | 1.65 |
| 550 | 77 | 8 | 4 –16 | 2.66 |
| 600 | 69 | 12 | 8 –16+ | — |

## Discussion

The minimum requirements for mercury concentration are computable from the chemical equation which appears to proceed to 86% completion. The amount of KI can be varied somewhat although 2.5 moles per mole of $HgI_2$ is near optimum for maximum color intensity. Low levels of nitrogen can be measured in the ultraviolet region if the mercury concentration is held below its interference levels. On the other hand high levels of nitrogen can be read at 600 mμ. It is possible that the over-all range could be extended slightly in each direction. In the low nitrogen region, difficulty was found with slow formation of color, and attention must be given to adequate reaction times.

The working range of Nessler's reaction at any single wavelength is relatively restricted. On some instruments, it is best to read at the wavelength that properly fits the nitrogen value and compute from a series of curves or conversion constants. For example, a method designed for 500 mμ will not accurately determine a nitrogen content of less than 2.0 μg., and it would be necessary to use a shorter wavelength to obtain the correct result. The relative error (*5*) calculated for 2.0 μg. of N per milliliter is 3.2% for a 1% photometric error; for 1.0 μg. of N the error becomes 7.2%, and at .5 μg. it is 11.0%. Knowledge of the working range of Nessler's reaction is useful in avoiding repeat determinations when the sample does not come within the prescribed limits.

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# Improvements on the Automatic Determination of Micro Amounts of Serum Calcium

*H. V. Malmstadt and T. P. Hadjiioannou*

**Calcium in 0.2 ml. of blood serum can be titrated automatically in about 10 sec. with a reproducibility of 1–2%. It is not necessary to deproteinize the serum, and the complete procedure requires less than 1 min.**

RECENT WORK has shown that calcium in 0.2 ml. of blood serum can be titrated automatically in less than 10 sec. with a reproducibility of 1–2%. It is not necessary to deproteinize the serum, so the total procedure requires less than 1 min. This new procedure is presented because it decreases both the quantity of serum and analysis time per determination to one-fifth or less those for the previously described procedure (*1*).

## Reagents

All solutions are prepared with deionized water.

*Standard calcium solution (0.00250 M)* Dissolve 0.2502 gm. dry A.R. $CaCO_3$ and approximately 0.24 gm. $MgSO_4 \cdot 7H_2O$ in 50–55 ml. 0.1 HCl and make up to 1 L. with water.

*EDTA solution* Dissolve 1.875 gm. of disodium salt of ethylenediaminetetraacetic acid (EDTA) in water and make up to 1 L. with water. Standardize this solution against the standard calcium solution and store in a polyethylene bottle.

*Calcon solution* Dissolve 0.020 gm. Calcon (Cl 202, Baker) in 25 ml. of methanol.

*Sodium hydroxide, 0.3 N aqueous solution* Stored in a polyethylene bottle.

*Potassium cyanide, 2% (w/v) aqueous solution*

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## Procedure

### Automatic Titrator

The titrator* used was the same as previously (*1*) except for the following. A plastic holder for an 18-mm. o.d. test-tube titration cell was made as shown in Fig. 1, and mounted on the beaker platform.

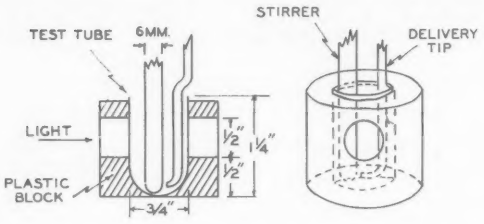


**Fig. 1.** Holder for test-tube titration cell.

Optimum focusing of the light was obtained by adjusting the position of the light source so that the center of the light beam passed through the test tube and fell on the detector lens mounted on the right side of the "spectro" compartment. A 6-mm. glass rod stirrer and the micro delivery tip were positioned as shown in Fig. 1. It is important that the stirrer be positioned 2–4 mm. from the bottom of the tube and the delivery tip *next to* and at the end of the stirrer. Also, a direct-reading, constant-rate, motor-driven buret† was used with the 10-ml. barrel, which provides a titrant delivery rate of 1 ml./min. The buret is plugged into the valve outlet on the back of the "spectro-electro" unit. Since only about 0.1 ml. of titrant is used for an average sample, 80–100 titrations can be performed without refilling the buret.

A red-cellophane cut-off filter is used in the auxiliary filter holder together with the usual 650-m$\mu$ interference filter which is dialed into position.

### Titrant Standardization

Pipet 0.200 ml. ($V_{Ca}$) of standard calcium solution into the tube, and add 2.0 ml. of 0.3 N NaOH and one drop of Calcon indicator. Push the start button on the control unit; this starts only the stirring because the buret drive is in "off" position. After 10–15 sec. of mixing, turn on the constant-rate buret. The titrant delivery is stopped automatically at the end point and the dial reading, $R$, is recorded. (The

*Sargent-Malmstadt Spectro-Electro titrator, E. H. Sargent & Co., Chicago, Ill.
†Model S-11120-1, E. H. Sargent Co.

volume of titrant delivered is $2R/1000$ ml.) Empty the reaction vessel by inserting a polyethylene aspirator tube and drawing out the contents. Follow with rinsing and withdrawal of rinse solution. Prior to the next titration reset the buret dial to zero and turn the drive switch to "off."

Repeat the titration above using 0.100 ml. of standard calcium solution and record the dial reading $R_1$. The blank, $B$, of the titration and the molarity $M_T$ of EDTA titrant are calculated by equations (1) and (2), respectively:

$$2R_1 - R = B \tag{1}$$

$$M_T = \frac{V_{Ca}\, M_{Ca}}{V_T} = \frac{0.200\ (0.0025)}{0.002\ (R - B)} = \frac{0.250}{R - B} \tag{2}$$

where $V_T$ is actual volume used for titration of 0.2 ml. of standard calcium solution.

### Determination of Calcium in Serum

#### Procedure 1

Follow the same procedure given for the standardization of EDTA solution but use 0.200 ml. of serum ($V_S$) instead of the standard calcium solution. Record the dial reading, $R_S$ and calculate the calcium as milligrams Ca per 100 ml. of serum, using Equation 3, or more simply by using Equation 3a.

$$V'_T M_T F_{Ca} \left(\frac{100}{V_S}\right) = 0.002\ (R_S - B)\ M_T\ (40.08) \left(\frac{100}{0.200}\right) =$$

$$40.08\ M_T\ (R_S - B) = \text{mg. Ca/100 ml. serum.} \tag{3}$$

If $M_T = 0.00500$ M, Equation 3 becomes

$$(R_S - B)\ (0.200) = \text{mg. Ca/100 ml. serum.} \tag{3a}$$

#### Procedure 2

If the phosphate content of the serum is abnormally high, titrate a mixture of 0.100 ml. serum and 0.100 ml. standard calcium solution according to the procedure given for the standardization of titrant, and record the dial reading $R'_S$. The volume of titrant equivalent to the calcium added to the serum is $0.00025/M_T$ ml. Calculate the serum calcium by Equation 4, or more simply by Equation 4a.

$$\left[0.002\ (R'_S - B) - \frac{0.00025}{M_T}\right] M_T\ (40.08) \left(\frac{100}{0.100}\right) = \left[2(R'_S - B)\ M_T - 0.25\right]$$

$$(40.08) = \text{mg. Ca/100 ml. serum} \tag{4}$$

If $M_T = 0.00500$ M, Equation 4 becomes

$$(R'_S - B - 25)\ (0.400) = \text{mg. Ca/100 ml. serum.} \qquad (4a)$$

## Results and Discussion

A typical set of data for 11 serum control samples, from four manufacturers, is presented in Table 1. Results are reproducible within 1–2%. The average value determined for each sample was well within the manufacturer's permissible variation, which was given as ± 2% (Sample 9) to ± 5% (Samples 1–5). Recovery studies were made on the same samples to provide an additional check on the method: 0.100 ml. of standard calcium solution was added to 0.100 ml. of serum by means of a microliter syringe. The recovery of Ca varied from 97 to 103%, with an average of 100.8%. The same precision and recovery were obtained when the method was applied to a large number of human blood serum samples. The method was tested by 21 students as part of an experiment, and after 1 hr. of instruction about the procedure and instrument, they obtained results reproducible within 2%.

When several bottles of EDTA were used, the variation in concentration by direct weight was less than 0.5%, the titrant was 0.00500 M, and the simplified equations 3a and 4a were used for calculations. However, in one case, the assay for EDTA was less than 98%. Therefore, the standardization step is recommended to determine the purity of a bottle of EDTA. Subsequently the appropriate amounts of EDTA can be weighed to prepare a 0.00500 M EDTA solution so that equations 3a and 4a can be used for calculations.

With the special microholder and reaction vessel, it is necessary to

**Table 1.** Automatic Titration Results for 0.2-ml. Aliquots of Serum Control Samples

| *Sample* | *Calcium (mg./100 ml. serum)* | |
|---|---|---|
| | *Reported* | *Found* |
| 1 | 10.2 | 10.1, 10.2 |
| 2 | 10.0 | 10.2, 10.3 |
| 3 | 9.9 | 9.9, 9.7 |
| 4 | 7.2 | 7.4, 7.3 |
| 5 | 7.0 | 7.1, 7.1 |
| 6 | 10.2 | 10.2, 10.3 |
| 7 | 9.8 | 9.9, 9.8 |
| 8 | 9.1 | 9.0, 8.9 |
| 9 | 8.8 | 8.9, 8.7 |
| 10 | 11.6 | 11.8, 11.6 |
| 11 | 11.8 | 11.8, 11.6 |

focus the light so that the center of the incident beam is centered on the solution and a sufficient amount of light falls on the detector. Differences in the focusing of the light and in the position of the stirrer and delivery tip might lead to differences in the value of blank. The blank can be determined precisely by titrating several 0.2-ml. aliquots and 0.1-ml. aliquots of the standard calcium solution. Over a period of several weeks the blank was 0.014 ± 0.001 ml.

When calcium solution containing no magnesium was used as standard, the blank was smaller by about 0.001 ml. as compared with serum samples or calcium solution containing small amounts of magnesium. It seems that the end point is retarded slightly in the presence of magnesium hydroxide. Therefore, magnesium is added to the standard calcium solution in amounts approximately equivalent to those found in an average blood serum sample to duplicate more closely the conditions present in serum samples.

When abnormally high concentrations of both calcium and phosphate are present, either premature end-points are obtained, or, sometimes, there is no automatic termination at the end point. This is caused by precipitation of calcium phosphate at the high *p*H (about 13) of the solution. In such cases only 0.100 ml. of serum is used for the titration. However, more accurate and precise results were obtained when 0.100 ml. of standard calcium solution was added to 0.100 ml. of serum (Procedure 2). Abnormal serum Samples 4 and 5 containing 8.2 and 8.0 mg. P-inorganic per 100 ml. serum, respectively, were analyzed by both procedures, and the results obtained agreed within ± 2%. Abnormal serum Samples 10 and 11 containing 12.4 and 7.1 mg. P per 100 ml., gave premature end points when Procedure 1 was used.

Addition of potassium cyanide solution to mask iron and copper was not necessary in the analysis of serum control samples and of several human serum samples. However, in hemolyzed serum, a drop of cyanide solution should be added and the solution stirred prior to the addition of Calcon indicator.

A red-cellophane filter is used instead of the yellow cut-off filter supplied with the instrument because it cuts out completely the radiation transmitted by the 650-m$\mu$ interference filter in the short wavelength region of the visible. However, the yellow cut-off filter can be used.

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# A Spectrophotometric Method for Measuring Serum Trypsin Inhibitor Capacity

*George M. Homer, Bernard J. Katchman, and Robert E. Zipf*

**The spectrophotometric method of Schwert and Takenaka (11) for the assay of trypsin esterolytic activity has been modified for the measurement of the trypsin inhibitor capacity of serum. An average value of 0.99 mg. of trypsin inhibited per milliliter of serum (3 S.D. = ±0.30) was determined for 50 control subjects. Patient serum assay values determined with this method have been found similar to those determined with the modified Kunitz method using $I^{131}$-labeled casein as the substrate—in the normal range for a variety of pathologic conditions and in the elevated range for abnormalities of the pancreas and generalized cancer conditions. From the standpoints of accuracy, simplicity, and speed this technic is believed to be an improvement over previous methods for the measurement of this serum variable.**

THE NEED for additional blood tests for the diagnosis of pancreatic dysfunction, coupled with our own interest in diagnostic enzyme studies resulted in previous work in this area (*1, 2*). A continued interest in the serum trypsin inhibitor values in cases of malignancy has further stimulated the development of a less complex but more accurate procedure than that using $I^{131}$-labeled casein as the substrate.

Previous methods used for the measurement of this serum variable include the following: the use of gelatin as a substrate, by Hussey and Northrop (*3*); a refractometric method using casein as a substrate, by Grob (*4*); an assay based on the dissolution of a blood clot, by Clark *et al.* (*5*); or hemostatic globulin in place of blood, by Christensen (*6*); fibrin tagged with $I^{131}$ as a substrate, by Shulman (*7*); and the spectro-

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photometric technic of Kunitz (*8*), by Jacobsson (*9*) and Bundy and Mehl (*10*).

The basis for most of these methods is that trypsin activity measured in terms of substrate hydrolysis is linear with enzyme concentration. A known, excess amount of crystalline trypsin is mixed with serum, and the enzymatic activity of that trypsin which is not complexed by the serum inhibitor is measured. Comparison with the activity of the known crystalline trypsin solution in the absence of serum provides a means of estimating the serum inhibitor capacity.

Time, temperature, and *p*H are 3 important factors which must be considered in the evaluation of a method for the measurement of trypsin activity. Trypsin activity is optimum at about *p*H 8, but above *p*H 6 trypsin solutions are inactivated by autolysis. In the casein methods (*1, 10*), the loss of trypsin activity may occur since trypsin is exposed to a *p*H of 7.0 or 7.6 over a 30-min. incubation period at 37° which increases the rate of autolysis. When measuring trypsin activity either with the casein method (*8*) or that using an artificial substrate (*11*), the reaction mixture may be made up to a constant volume with 0.0025 M HCl with a minimal loss of enzyme activity. However, if constant volume is attained with a phosphate buffer (*p*H 7.0), trypsin activity is reduced even when calcium is added to increase the stability of the trypsin solutions. A further consideration in the measurement of the serum trypsin inhibitor capacity (TIC) is the fact that serum inhibitory activity disappears on exposure to a *p*H 6 or less (*10*). In effect, the relationship which must be considered is the instability involving autolysis of trypsin at higher *p*H and the instability of the serum inhibitors at lower *p*H.

In the method reported here, these difficulties have been reduced to a minimum with the development of a simple, but still accurate, technic for the measurement of this serum variable. The spectrophotometric method of Schwert and Takenaka (*11*) for the assay of trypsin esterolytic activity has been adapted to this purpose. The time of exposure of trypsin to solutions of *p*H above 3 has been reduced to 2–3 min. from the 30-min. incubation required for the casein method (*1*). In addition, the reaction temperature has been reduced from 37° to 27°. More similar experimental conditions are thus provided for trypsin and trypsin-serum assays while still attaining maximal trypsin activity. This procedure is also more versatile since the assay of a single trypsin-serum tube provides information for the correct serum dilution within a matter of a few minutes. The longer casein procedure provides this infor-

mation only after lengthy manipulations, and unless different dilutions have been made on a single serum, a repeat assay may be required.

## Experimental

### Reagents

*Trypsin** (*lyophilized-crystalline*) A solution of approximately 425 μg./ml. in 0.0025 M HCl.

*Soybean trypsin inhibitor** (*crystalline*) A solution of approximately 175 μg./ml. 0.0025 M HCl.

*Substrate* Benzoyl-l-arginine ethylester hydrochloride (BAEE),† 0.50 mM in 0.1 M phosphate buffer (It may be stored in the refrigerator.)

*Hydrochloric acid* 0.0025 M, *p*H 2.6.

*Phosphate buffer* $Na_2HPO_4$-$KH_2PO_4$, 0.1M, *p*H 7.0.

*Serums* Diluted 1:5 or 1:10 in 0.1M phosphate buffer

### Procedure

A Beckman, Model DK recording spectrophotometer was used. The temperature of the sample chamber was maintained relatively constant (27° ± 2.0) through the use of the lamp-housing cooling system and by leaving the cover off the sample chamber when a sample was not being measured.

For all studies on the serum TIC, trypsin and soybean trypsin inhibitor (STI) solutions were prepared fresh daily. The protein concentration of these solutions was determined by measuring the absorbancies at 280 mμ and then multiplying by the conversion factors 0.585 and 1.10 for trypsin and STI, respectively (*8*). All glassware and pipets should be acid-washed following the usual detergent wash.

#### Enzyme Assay

A trypsin activity measurement over a range of trypsin concentration is not ordinarily made when assaying for serum TIC. This measurement is not required for the serum assay as is the enzyme standardization measurement which is described below. The assay of the enzyme is easily effected, however, in the following manner. Three milliliters of BAEE substrate are pipetted into the control and the test cuvets, and the spectrophotometer is balanced at 253 mμ. The cuvets

*Worthington Biochemical Corporation, Freehold, N. J.

†Mann Research Labs, New York, N. Y.

and holder are removed from the instrument and 0.2 ml. of 0.0025 M HCl is added to the control cuvet, which is inverted with the aid of a parafilm cover. Increasing amounts of trypsin (0.1, 0.2, 0.3 ml. of the 425 $\mu$g./ml. solution) are pipetted into 10 $\times$ 1.5-cm. test tubes. Volumes

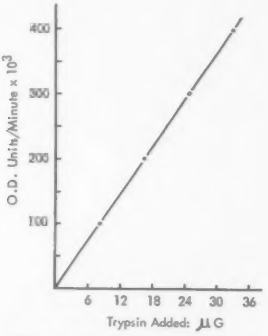


**Fig. 1.** Trypsin assay curve.

are made up to 1.0 ml. with 0.0025 M HCl and the contents mixed by shaking. In rapid succession, a 0.2-ml. volume from a tube is added to the test cuvet, and as mixing by inversion is initiated, a stopwatch is started. The cuvet is returned to the holder, and absorbance readings are taken every 10 sec. for 1–2 min. The reaction is linear over this period with the rate of esterolysis proportional to enzyme concentration. An enzyme assay curve is shown in Fig. 1 where the rates of reaction, as absorbance units per minute, are plotted against enzyme concentration as microgram of trypsin.

### Standardization of Enzyme

With the concentration of trypsin as given (425 $\mu$g./ml.), a 0.3-ml. volume, assayed as above, provides a rate of hydrolysis of approximately 0.3 absorbance unit per minute. This single-point assay of the enzyme is made with each standardization and repeated with alternate serum assays. A series of tubes is prepared, each containing 0.3 ml. of trypsin (425 $\mu$g./ml. solution) and increasing amounts of STI (0.1, 0.2, 0.3, and 0.4 ml. of 175 $\mu$g./ml. solution) with sufficient 0.0025 M HCl to make 1.0 ml. The solutions are assayed as explained above and the rates of substrate hydrolysis determined. In Fig. 2 a typical enzyme standardization curve is presented. The rates of substrate hydrolysis are plotted against milliliters of STI used in the assay. A straight line

drawn through the points will cross the abscissa at the theoretical volume of STI solution which will completely inhibit the enzymatic activity of 0.3 ml. of trypsin. As shown in the plot, 0.59 ml. of STI solution completely inhibited the trypsin. By proportion, 1.96 ml. of STI solu-

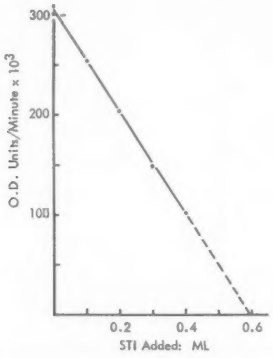


**Fig. 2.** Trypsin standardization curve.

tion would inhibit 1.0 ml. of this trypsin solution. Using the concentration of STI solution as determined with the spectrophotometer (absorbance times conversion factor, assumed for sample calculation as equal to 175 $\mu$g./ml.), the microgram amount of STI is determined: 175 $\mu$g./ml. $\times$ 1.96 ml. = 348 $\mu$g. Assuming a trypsin-STI weight-combining ratio of 1:1, the trypsin solution is standardized as equal to 348 $\mu$g./ml. The experimental trypsin-STI ratio for this example would be the trypsin solution concentration, as determined with the spectrophotometer and conversion factor, 425 $\mu$g./ml., divided by 348 $\mu$g./ml. for a ratio of 1.22:1.

### Serum Assay

The assay of serum is carried out essentially in the same manner as the enzyme standardization. A constant amount (0.3 ml.) of trypsin solution is mixed with increasing amounts (0.2, 0.3, 0.4 ml.) of diluted serum, made up to 1.0 ml. volume with 0.0025 M HCl, and assayed as above. In actual practice, trypsin and the requisite amount of HCl are added first, followed by the serum, one tube at a time, with assay of the sample immediately following. This is done to minimize the time that the trypsin is exposed to the higher *p*H of the phosphate buffer and substrate solutions. A serum assay curve is shown in Fig. 3 where the

rates of substrate hydrolysis have been plotted against the amount of diluted serum. A straight-line relationship is observed to approximately 80% inhibition of the added trypsin. The line is projected linearly to the abscissa and the point of crossing used in the calcula-

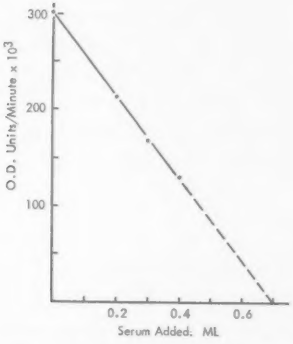


**Fig. 3.** Serum assay curve.

tions. In the example cited (Fig. 3), 0.70 ml. of serum would theoretically completely inhibit the 0.3 ml. of trypsin which was used in the test. By proportion, 2.33 ml. of serum would completely inhibit the enzymatic activity of 1.0 ml. of the trypsin solution. Since the trypsin was standardized in terms of STI, we then equate serum and the standardized trypsin solutions such that 2.33 ml. of serum inhibits 348 $\mu$g. of trypsin, or 1.0 ml. serum inhibits the activity of 150 $\mu$g. of trypsin. On the basis of a 1:10 dilution of the serum, the final trypsin inhibitor capacity is expressed as 1.50 mg. of trypsin inhibited per milliliter of serum.

## Results and Discussion

### Standardization of Enzyme

The weight-combining ratio of trypsin and STI was taken as 1:1 by Kunitz (*8*) on the basis of his molecular-weight determinations for the components. Other investigators (*12, 13*) have confirmed this work although reporting slightly lower molecular weights for trypsin and STI. In our previous report (*1*), trypsin solutions were standardized on the basis of a 1:1 ratio between trypsin and STI, and the data reported here have been treated similarly. This is done even though the combining ratios in our studies (using the conversion factors 0.585 and

1.10 for trypsin and STI, respectively) have averaged 1.22:1. The clinical usefulness of the test is in no way dependent upon the variation between the molecular weights of trypsin and STI. If further investigation should establish a weight-combining ratio other than 1:1, our data could easily be corrected by the use of a constant. Although the standardization procedure functions as a means of correcting for variations in trypsin activity, it also serves to maintain an additional check on the assay. If the trypsin-STI ratio is found to be outside the range of 1.16:1 to 1.28:1, the solutions are discarded, fresh solutions prepared, and trypsin restandardized. In this manner, the possibility of using a contaminated STI solution and of incorrectly evaluating a serum is reduced.

### Serum Assays

Serums from blood-donor subjects have been analyzed for TIC to determine the normal range for this method and to compare with other methods of assay. A mean value of 0.99 mg. trypsin inhibited per milliliter of serum (S.D. = ± 0.10 mg./ml.) was obtained for 50 persons classified as normal. This value is slightly higher than the value of 0.87 mg./ml. determined previously using $I^{131}$-labeled casein as the substrate (*1, 2*). The upper limit for so-called normal at a 3-$\sigma$ range with this method is the same for the previously reported method: 1.29 mg./ml.

In Table 1, the results are presented from a study comparing this method and the modified Kunitz method where $I^{131}$-labeled casein is used as the substrate. Values were found to be quite similar with values from the new method, ranging randomly on both sides of values from the modified Kunitz method. An analysis of these data for the coefficient of correlation indicated a high degree of relationship between the two sets of values.

As a means of evaluating the precision of this method, a single serum sample from a control was assayed, repeatedly, over a period of 35 days. The serum was stored in a freezer at −20° between assays. As shown in Table 2, a small range of 0.06 mg./ml. was found between the extremes of the 7 determinations with a standard deviation of ± 0.02 mg./ml.

The daily variation in serum TIC values was determined on 2 patients. Both patients were receiving a therapy which was believed to have no effect on the serum assay. The results of these analyses are also presented in Table 2. A range of 0.12 and 0.13 mg./ml., respective-

**Table 1.** COMPARISON STUDY BETWEEN TWO METHODS

| Serum No. | Trypsin inhibited (mg./ml. serum) | |
|---|---|---|
| | Modified Kunitz method | Modified Schwert method |
| 1 | 2.14 | 2.13 |
| 2 | 2.04 | 2.09 |
| 3 | 1.81 | 1.79 |
| 4 | 2.06 | 2.16 |
| 5 | 1.34 | 1.38 |
| 6 | 2.18 | 2.01 |
| 7 | 1.58 | 1.34 |
| 8 | 1.85 | 2.04 |
| 9 | 2.27 | 2.48 |
| 10 | 0.69 | 0.69 |
| 11 | 2.00 | 1.78 |
| 12 | 1.78 | 1.70 |
| 13 | 0.74 | 0.70 |
| 14 | 0.81 | 0.89 |
| 15 | 1.14 | 1.00 |
| 16 | 1.26 | 1.24 |
| 17 | 1.08 | 1.00 |
| 18 | 0.94 | 0.94 |
| 19 | 0.99 | 0.90 |
| 20 | 1.32 | 1.24 |

Correlation Coefficient, r, = 0.97.
Table r at the 1% point = 0.56.

ly, were found between the extremes of the determinations for the 2 subjects. Standard deviations were found to be only twice that determined for repeated assays of a single sample: ± 0.04 mg./ml.

A total of 73 separate serums from hospital patients have been assayed for the serum TIC since our previous reports on this procedure (*1*, *2*). Of this number, 21 were found to have values in the normal range. Some of the diagnoses for these cases are as follows: diabetes mellitus; acute thrombophlebitis, left lower extremity; myocardial

**Table 2.** PRECISION OF METHOD AND DAILY VARIATION IN SERUM TIC VALUES

| Subject | No. determinations | Time interval (days) | Trypsin inhibited (mg./ml. serum) | | S.D. (mg./ml.) |
|---|---|---|---|---|---|
| | | | Average | Range | |
| Control (single serum sample) | 7 | 35 | 0.87 | 0.84–0.90 | ±0.02 |
| Patient A (separate serum samples) | 8 | 20 | 1.03 | 0.98–1.10 | ±0.04 |
| Patient B (separate serum samples) | 11 | 25 | 0.86 | 0.80–0.93 | ±0.04 |

**Table 3.** TRYPSIN INHIBITOR CAPACITY OF SERUMS FROM PATIENTS' ELEVATED VALUES

| *No. subjects* | *Diagnosis* | *Trypsin inhibited (mg./ml. serum)* | |
|---|---|---|---|
| | | *Average* | *Range* |
| 10 | Pancreatitis | 1.58 | 1.32–2.10 |
| 4 | Cystic fibrosis | 1.51 | 1.42–1.58 |
| 5 | Cancer of pancreas | 2.40 | 1.67–3.27 |
| 20 | Cancer of liver, G. I. tract, G. U. system | 1.97 | 1.33–2.88 |

infarction; arteriosclerotic heart disease; psychoneurosis; hiatal hernia and esophagitis; and epigastric pain of unknown origin. In general, the diagnoses for this group of hospital patients are similar to those previously studied.

Elevated values were found, as previously, to be largely associated in patients with abnormalities of the pancreas and with all types of cancer. The data from these studies are presented in Table 3, where the diagnoses have been classified into several general categories. Average values have been determined for the separate groups, and as may be observed, conditions of cancer of the pancreas resulted in the highest TIC values. Generalized cancer conditions also showed high serum inhibitor values with an average value for all patients above the average values for pancreatic involvement other than cancer of the pancreas (acute or chronic pancreatitis or cystic fibrosis).

Elevated values have been found to a small degree for cases which, according to the physician's diagnosis (as taken from the patient's chart), did not involve pancreatic dysfunction or a cancer condition. Blood samples were obtained from the laboratory without regard to previous medication. As a result, the influence of various drugs on the serum TIC determination has not been evaluated, and some of these elevated values may have been a result of a specific type of medication. The progress of patients has not been followed to determine if a specific elevated serum TIC value was a false-positive or actually predicting a condition not in evidence by means of other tests. The results so far indicate that more definite information is necessary before attempting to clarify the meaning of serum-trypsin inhibitor values in some cases.

The information in Table 4 provides a summary of all data, published and unpublished to the present. As discussed above, a perfect correspondence between elevated values according to the serum assay and patient diagnoses has not been found. The discrepancy exists, to

the largest degree, where elevated values have been found in cases not involving an abnormality of the pancreas, cancer of the pancreas, or other types of cancer. This grouping constitutes the second classification in this table (Type 2 error). The Type 1 error for the total data analysis, which involves normal serum TIC values in cases where a

**Table 4.** TRYPSIN INHIBITOR CAPACITY OF SERUMS FROM PATIENTS' ELEVATED VALUES

| | |
|---|---|
| Number of serums assayed | 169 |
| Type 1 error: No. serums of normal value where diagnosis indicated pancreatic abnormality or cancer condition | 5 |
| % of total serums | 3 |
| Type 2 error: No. serums of elevated value where diagnosis indicated no pancreatic abnormality or cancer condition | 20 |
| % of total serums | 12 |

pancreatic disturbance or cancer condition was diagnosed, is rather small. This evaluation does not include a small number of cases diagnosed as pancreatic involvement in which enzyme therapy or antibiotic therapy for cystic fibrotic cases was in progress when the serum was assayed. These treatments would be expected to alter the clinical picture and, in the case of enzyme therapy, to interfere with the assay.

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the Clinical Chemist

# Committee on Standards and Control

## Volunteers Needed

A NUMBER of members of the Association have expressed interest in the programs of the Committee on Standards and Control to the extent of offering their services for group projects. It has taken some time to set up programs which can utilize this help effectively but there is now an opportunity for those who are interested in contributing their time and knowledge to projects which we feel can yield useful information.

We have been fortunate in being offered the assistance of the Heart Diseases Control Laboratory of the U. S. Public Health Service, which has opened its Cholesterol Standardization program to laboratories who choose to participate in our study. As planned, the volunteer laboratories would enroll in an abbreviated version of the H. D. C. L.'s normal program, with the results being processed in the usual way. The derived data, coded, would be made available to the Committee for its own evaluation and guidance in subsequent studies. Participation requires a serious effort directed toward ironing out difficulties in one's own routine cholesterol assays, followed by periodic runs of survey samples over a period of about six months. It thus offers an opportunity for self-evaluation now available to few laboratories. The total number of participants who can be handled is limited and the basis of selection is tentatively one which would yield the broadest regional representation among Association members.

The Committee would like to hear from clinical chemists interested in assisting with field studies in standardization of thymol turbidity assays. All expressions of interest and opinion on general problems of standardization and control will be appreciated. Communications may be addressed to: Alan Mather, Chairman, Committee on Standards and Control, Box 1548, Wilmington 99, Del.

## Section News

### Cleveland Section

The officers elected for 1963-1964 are as follows: Willard Faulkner, Chairman; Irving Sunshine, Vice-Chairman; Herbert Thompson, Secretary-Treasurer; Adrian Hainline, Representative to the National Executive Committee.

### Upstate New York Section

The officers elected for 1963-1964 are as follows: Theodore Peters, Jr., Chairman; Michael Vanko, Chairman-Elect; Nathan Radin, Secretary-Treasurer; Martin Murray, Executive Committeeman; and Royden Rand, Alternate Executive Committeeman.

On June 7, 1963, the Upstate New York Section held a meeting at the Tompkins County Memorial Hospital. The scientific session consisted of a panel discussing protein analysis. With Max Chilcote as moderator the Biuret Reagent was discussed by Anthony LaPaglia, the Kjeldahl Method was discussed by Martin Murray, the Ultraviolet Region and Refractometric Methods were discussed by Royden Rand, and "Odd-Ball" Protein Analyses were discussed by Nathan Radin.

### Connecticut Section

The officers elected for 1963-1964 are as follows: Pauline Hald, Chairman; David Seligson, Chairman-Elect; Santo J. Coco, Secretary-Treasurer; John F. Iannucci, Membership Chairman; George N. Bowers, Representative to the National Executive Committee.

### Philadelphia Section

The officers elected for 1963-1964 are as follows: Meyer Samson, President; Seymour Winsten, President-Elect; Margaret Ryland, Secretary-Treasurer; Carl Alper, Representative to the National Executive Committee.

The ChemiCliniC held in April dealt with Cross-Contamination in the Determination of Serum Protein-Bound Iodine. The speaker was Mr. Meyer Samson, of The Samson Laboratories. Two short papers were presented at the May meeting. Dr. Leonore H. Koehler spoke on "Measurement of Adenosine Deaminase and its Clinical Application." Dr. Seymour Winsten spoke on the "Continuous Flow Electrophoresis of Serum Proteins with Micro-glass Beads."

### Southern California Section

The officers elected for 1963-1964 are as follows: Norman D. Lee, Chairman; James A. Demetriou, Program Chairman and Chairman-Elect; Solly Notrica, Secretary-Treasurer; Herbert O. Carne, Rex E. Sterling, Clyde A. Dubbs, Membership Committee.

During the past season seven major programs were given. In October Dr. Edward Arquilla spoke on "Immunological Techniques for Detection of Hormones and Their Antibodies." Dr. Arquilla is associated with UCLA Medical School. In November Dr. Daniel Simmons, UCLA Medical School, spoke on "Pulmonary Mechanisms," and Dr. Jack Hackney, Loma Linda University, spoke on "Renal

Mechanisms." In January Dr. Willard Vander Laan, Scripps Clinic and Research Foundation, spoke on "Thyroid Stimulating Hormone." At the February meeting Dr. Michael Schotz, V.A. Center and UCLA Medical School, spoke on "Employing Thin Layer Chromatography; and Dr. David Blankenhorn, USC Medical School, spoke on "Employing Gas Chromatography." In March Dr. Jack Lieberman, Long Beach V.A. Hospital, spoke on "Proteolytic Enzymes in Respiratory Secretions Relative to Cystic Fibrosis"; and Dr. Morton Grossman, V.A. Center and UCLA Medical School, spoke on "Internal Secretions of the Pancreas in Cystic Fibrosis." A Symposium on Calcium was held in April—Dr. Charles Kleeman, UCLA Medical School, "Relationships Between Parathyroid Gland and Calcium Metabolism"; Dr. Milton Rubini, V.A. Center and UCLA Medical School, "Relationships Between Renal Physiology and Calcium Metabolism"; and Dr. John Bethune, USC Medical School, "Ultrafilterable Calcium in Plasma." A Research Symposium was held in May—Dr. Clyde Dubbs and Edward May of St. John's Hospital and UCLA Medical School, "Gel Electrophoresis of Prestained Serum Lipoproteins"; Dr. George Stevenson, Bio-Science Laboratories, "Determination of Theophylline in Blood"; Dr. William Webster, Jr., Clinical Laboratory Medical Group, (presented by Dr. William Kern), "Simplified Colorimetric Method for Determination of Urinary LDH with Clinical Evaluation"; George Kingsley, Roscoe Schaffert, Gloria Getchell, and T. E. Jones, V.A. Center and UCLA Medical School, "Investigation of Tissue Arsenic in Human Malignancy"; and Dr. A. A. Fernandez, Charles Sobel, and Dr. S. L. Jacobs, Bio-Science Laboratories, "Catalytic Method for Colorimetric Determination of Submicrogram Quantities of Manganese."

FIFTH INTERNATIONAL CONGRESS
ON CLINICAL CHEMISTRY*
August 19-23, 1963 Detroit, Mich.

# Abstracts of Papers from Scientific Sessions

## *MONDAY, AUG. 19*

### *SECTION A: AUTOMATION*

**1. Methods for the automated determination of serum phosphate.** E. Epstein, E. S. Baginski, and B. Zak (*Departments of Pathology, St. Joseph Mercy Hospital, Pontiac, Mich.; Sinai Hospital and Wayne State University College of Medicine, Detroit, Mich.*).

Three methods are presented for the automated determination of serum phosphate, each of which possesses features which may recommend them for routine and special analytical problems. In Method 1, ascorbic acid–reduced phosphomolybdate is stabilized by a citrate-arsenite mixture, yielding a sensitive reaction. In Method 2, the molybdate solution contains both Mo(VI) and Mo(V), where the phosphomolybdate formed is reduced by the same reagent. In Method 3, a combined vanadomolybdate reagent reacts with phosphate to form a yellow complex measurable in the near ultraviolet.

Method 1 has the advantage of being the most sensitive of the three: the reagents are stable, the calibration is linear, and the color formed is stable.

Method 2, the Zindazde reaction, is self-reducing with heat, sensitive, and possesses a minimal blank.

Method 3 also has a single color development reagent, develops color at room temperature without a reducing agent, and calibrates linearly.

Data will be presented to show that the three reactions agree with each other, recover phosphate equally well from serum, and correlate analytically with manual phosphate procedures.

*Organized by The American Association of Clinical Chemists and The Canadian Society for Clinical Chemistry.

**2. An automated procedure for the determination of phosphorus and alkaline phosphatase (Bodansky) in serum.** Norbert W. Tietz and Allen Green (*Mount Sinai Hospital, Chicago 8, Ill.*).

An automated modification of the Bodansky method for alkaline phosphatase and phosphorus in serum is presented. The tests can be performed at a rate of 40 tests per hour. Statistical evaluation showed that the methods are accurate and reproducible.

The normal values for this method were found to be 1.3–4.0 U., with a mean of 2.5 ± 0.59 S.D.

An alternative procedure is also presented which has an increased sensitivity and which eliminates contamination by specimens high in alkaline phosphatase activity, but which can be run at a speed of only 20 tests per hour.

**3. A photometric system for automatically reading concentration.** D. K. McKay, D. Seligson, and W. R. Jewett (*Grace-New Haven Community Hospital and Yale University School of Medicine, New Haven 4, Conn.*).

Spectrophotometric or colorimetric data are normally obtained in some type of photometric unit which must be dealt with mathematically to provide a final result expressed in concentration. Such mathematical operations are time-consuming, require data transfer, and are subject to human error. A photometric system has been devised which can be adjusted to perform calculations automatically and provide direct readout of concentration. This system combines logarithmic conversion of %T with control of both $m$ and $b$, constants in the straight-line equation, $y = mx + b$, in which $y$ is absorbance, $m$ is slope, $x$ is concentration, and $b$ is the blank correction. The system also provides linearizing compensation for those reactions which do not follow the Beer-Lambert Law.

Concentration data are presented in digital form. Comparison of this system and the usual colorimetric technics and associated calculations are presented for linear and nonlinear procedures in order to demonstrate the accuracy, efficiency, and convenience of the direct-readout system.

The calibration procedure for the system is described, using a complete analysis as an example.

**4. Multiple automatic sequential analysis. I. Theoretical considerations.** Leonard T. Skeggs (*Department of Medicine and Surgery, Veterans Administration Hospital, and Department of Pathology, Western Reserve University, Cleveland, Ohio.*).

Certain automatic analytical methods (AutoAnalyzer) can be combined within a single-flow system to produce great savings in equipment, operator time, and sample consumption. The results of each of the different methods appear on

different recorders. The records must be individually calculated and the results for each patient collected before a report can be sent to the requesting physician. This problem was attacked by the construction of a multiple-flow system which permitted delivery of the several final colored streams to a colorimeter having multiple flow cells but a single optical path. Time delay coils were used to cause the color produced by a given sample for each of the various tests to reach a steady state at the colorimeter in a regular predetermined sequence. By mechanically shifting the colorimeter cells into the light path in the same sequence, a recording of signals for each test was automatically obtained. A recorder with a logarithmic slide wire was used so that pen motion was directly proportional to concentration. The recorder balancing motor was used to drive an adjustable retransmitting slide wire which controlled a second recorder carrying paper calibrated for each of the tests. Signals obtained from standard solutions were used to precalibrate the entire system. Thus a multiple, sequential, automatic analyzer has been produced in which the results of several determinations conducted on each sample appear on a single record in calculated form.

**5. Multiple automatic sequential analysis. II. Construction and operation of an eight channel instrument.** Leonard T. Skeggs (*Department of Medicine and Surgery, Veterans Administration Hospital, and Department of Pathology, Western Reserve University, Cleveland, Ohio*).

A multiple automatic analyzer has been constructed which determines albumin, total protein, chloride, carbon dioxide, sodium, potassium, glucose, and BUN. The results for each of these determinations are obtained in sequence for an individual sample, and are recorded on a single piece of paper. The entire system is standardized by means of a single multiple-standard solution containing all eight of the materials under test. Results appear on the final record in calculated and instantly usable form. If desired, the output signal may be used to activate a print-out device rather than a recorder. The signal is suitable for use with punch cards or other means of data handling. The equipment has been tested, and the results compared with standard single analyzer (AutoAnalyzer) systems under actual hospital operating conditions. The results of these comparisons suggest that the precision of the multiple system compares favorably with that of individual instruments, and that it possesses the great advantage of eliminating manual errors in calculation and in transcription of data. Use of the multiple system of analysis should reduce the cost and increase greatly the number and frequency of laboratory determinations. This should be of value not only in the laboratory diagnosis and treatment of patients, but also in the screening of the general population and in the establishment of individual norms.

## *SECTION B: METABOLIC (VARIOUS)*

**6. Uptake of aromatic amines by blood platelets.** D. V. Siva Sankar and D. Barbara Sankar (*Creedmoor State Hospital, Jamaica 27, N. Y.*).

The uptake of labeled serotonin and histamine by platelets was investigated in children hospitalized in the Creedmoor State Hospital. Blood was collected with heparin (100 U./ml. blood) in siliconized glassware, and platelet-rich plasma was prepared from the blood by centrifuging at 50 g. for 45 min. Then, 2–3 ml. of the plasma was incubated with 0.1 ml. (0.1 $\mu$M) of serotonin in 0.9% saline and 0.05 ml. (equal to 0.25 $\mu$c) of a solution of serotonin-2-$C^{14}$ (sp. activity, 6.75 $\mu$c/mM) for one hour at 37°. The platelets were isolated by centrifuging at 500 g. for 40 min. at 2–4° and washed twice with ice-cold saline. The final platelet pellet was lysed with 1 ml. water and by freezing and thawing. Since accurate platelet counts are impossible using the pellet, optical density readings at 660 m$\mu$ were used. The radioactivity in the lysate was counted using a GM counter. The uptake of serotonin was expressed as cpm/O.D. $\times$ 1000. These studies show that: (1) the uptake increases with increasing age in these children, (2) the uptake in autistic schizophrenic children is significantly less than in nonautistic children of comparable age, and (3) treatment with LSD-25 or UML-491 increases the uptake for the first 2 or 3 weeks, when the uptake returns to lower levels. Treatment with reserpine decreases the uptake, while imipramine had no consistent effect. Treatment with these drugs decreased histamine uptake. This technic of aromatic amine uptake may be of value in the clinical characterization of autism and possibly other diseases—e.g., thyrotoxicosis.

**7. Mauve factor and 6-sulfatoxy skatole: Two biochemical abnormalities associated with specific measures of psychiatric disease.** D. G. Irvine (*Department of Biochemistry, Saskatchewan Hospital, North Battleford, Canada*).

The biochemistry of mental illness has been approached through an interdisciplinary study emphasizing individual differences, empirical grouping of patients, the use of one group of patients as controls for another, and adherence to a rigid system of blinds and codes to make interdisciplinary exchange of study data impossible. These studies have demonstrated that patients differentiated on appropriate chemical grounds also show corresponding clinical differences.

For example, the excretion of mauve factor was found to be related to psychosis, perceptual disorder, "dissimulating" and psychotic personality profile, psychological organicity, partial disorientation, cooperative behavior, and abnormal EEG. Similarly, the excretion of 6-sulfatoxy skatole was associated with psychosis, paranoid personality profile, paranoid belligerent ward behavior, depression and hostility in interview, and relative macrocytosis.

Methods for the routine and large-scale extraction, purification, chromatog-

raphy, and partial characterization of mauve factor are described. Biochemical studies relating skatole to 6-sulfatoxy-skatole and other metabolites of psychiatric relevance are discussed, including the experimental production of these substances in rodents. Effects of antibiotics, dietary manipulations, and sources of error are assessed. Some implications of this general approach and these findings for the theory and practice of biochemical research in psychiatry are suggested.

**8. Serum lipids and cancer.** Harald von Elmendorff (*Medizinische Akademie, Dusseldorf, Moorenstrasse 5, Germany*).

The results of Haven and of Budwig were re-examined with gas-liquid chromatography. They found that the higher unsaturated fatty acids, among them the essential fatty acids, were diminished in the serum of cancer patients.

With improved extraction methods we found that, in cancer serum, arachidonic acid particularly is diminished, while the values for linolic acid were frequently normal, and the content of linolenic acid varied.

Chromatography on silica gel plates, according to Starek, showed good separation of serum phospholipids. The following fractions were noted: cardiolipin, colaminkephalin, lecithin, sphingomyelin, lysolecithin, and other lysoproducts. In normal serum the amount of lecithin found is several times greater than sphingomyelin. In cancer serum the lecithin-sphingomyelin ratio was shifted toward the sphingomyelin. This was confirmed with paper chromatography on silica gel paper. The sphingomyelin fraction contains other, not yet defined, substances poorly soluble in chloroform.

Column chromatography of the neutral fats on florisil showed the amount of cholesterol esters strikingly increased in the blood of cancer patients. This is in contrast to all other findings. It was found that this fraction contained not only cholesterol esters, but another compound, constituting more than 50% of the fraction. This compound could be found only in traces in normal serum, and is insoluble in acetic acid. It can be demonstrated on paper chromatograms of unpurified phospholipids, and has an RF value of 0.85.

**9. The effect of X-ray irradiation on the desoxyribonucleic acids of the spleen and radiosensitive tissue.** H. P. Schwarz, H. Desher, M. Hainski, and R. Lumley Jones (*Biochemistry Laboratory, Philadelphia General Hospital, Philadelphia, Pa.*).

Spleen DNA from whole-body–irradiated rats and controls has been studied with anion exchange chromatography, infrared spectroscopy, and electrometric titration. These studies showed that ionizing irradiation at 1000–3000 r caused an increase of a low-molecular DNA fraction eluted from ECTEOLA-cellulose at neutral *p*H to up to 2 times the control value. Infrared spectroscopy similarly

indicated such an increase of low-molecular DNA by showing a definite decrease of the extinction coefficients of the band at 970 cm.$^{-1}$ (10.3 $\mu$), which is caused by the internucleotide (P-O-$C_5'$) linkages in the irradiated DNA. Preliminary results of electrometric titrations indicate some breaks of hydrogen bonds in irradiated DNA and are suggestive of separation of the helixes following the exposure to X-rays.

**10. Surgical trauma and arylsulphatase activity of blood and the healing wound.** L. M. Dzialoszynski, J. Nowak, and A. Frohlich (*Animal Physiology Department, College of Agriculture, Poznan, Poland*).

Injury, such as surgical trauma, is followed by a number of metabolic changes, among them being retention of water and sodium and an increased excretion of nitrogen and potassium. It was also found that the activity of blood oxytocinase elevates as a result of operative trauma.

In this work the effect of surgical trauma on arylsulfatase activity in blood and in the healing wound was studied. Rabbits were used as experimental animals.

Six aseptic incisions in the skin and muscles, each about 5 cm. long, were made on the abdomen under local anesthesia. The edges of the first wound were cut out for enzyme estimations, and all wounds closed. On the second, fourth, sixth, tenth, and fourteenth day cuttings were taken from the second, third, fourth, fifth, and sixth wound, respectively, and arylsulfatase determined. The method of Roy, in which nitrocatechol sulfate serves as substrate was used for arylsulfatase estimations.

Blood arylsulfatase activity was examined before the operation and several days after and it was found that the enzyme activity increases by about 50% between the second and sixth day after the trauma.

Arylsulfatase activity of the skin and muscle is normally about one nitrocatechol U./mg. of wet tissue. In the healing wound, however, the mean activity during the first 2 weeks was 7 times higher in the skin and 14 times higher in the muscle. After 6 weeks the scars had normal arylsulfatase activity.

**11. Graded pilocarpine tests in the study of cystic fibrosis.** A. L. Tárnoky (*Royal Berkshire Hospital, Reading, England*).

The controversy on sweat electrolyte levels has recently been reviewed. Disagreement is limited to possible heterozygotes; conflicting values are mainly those of laboratories obtaining sweat by heat and those using chemical stimulation, the latter giving normal, the former often raised levels. The theory that chemical (but not thermal) stimulation acts as stress would agree with findings of di Sant'Agnese *et al.*; it now seems that a simpler explanation will account for the difference.

A comparison of thermal (TH) with pilocarpine (PC) tests suggests that the standard dose (0.2%) of PC gives the true result in normals and in fully manifested cystic fibrosis but that some possible heterozygotes require a higher dose (2.0% PC) for a full response. In an illustrative family where an affected baby died (PC-$Cl^-$ 136 mEq./L. independent of dose), 4 of 5 siblings and both parents showed agreement between TH and PC-$Cl^-$ levels, unaffected by the dose of PC. The fifth sibling, who had the highest TH-$Cl^-$ level (83 mEq./L.), gave a 0.2% PC result of 65 mEq./L., which was raised to the TH level (89 mEq./L.) by 2.0% PC. The figures are not connected with rates of sweating. Further work will show whether the two-level test is a reliable case-finding procedure.

**12. Das auffinden eines neuen mono-pyrrols (porphobilinogen B) in porphyriaurinen.** H. Petermann and J. Wyllie (*Department of Chemistry, Queen's University, Kingston, Ont., Canada*).

**13. The induction of po.-phyria in rats by hexachlorobenzene administration.** J. Wyllie and H. Petermann (*Department of Chemistry, Queen's University, Kingston, Ont., Canada*).

## *SECTION C: LIVER DYSFUNCTION*

**14. Studies on severe familial idiopathic jaundice of infancy.** O. P. Gray and R. A. Saunders (*The United Cardiff Hospitals, Clinical Pathology, Llandough Hospital, Penarth, Glam, Wales*).

The case histories of two infant siblings with severe cholestatic jaundice with some features similar to those described by Rotor but with certain significant differences are reported. This disease, which is familial, has been fatal in one of the patients, and has progressed to give liver damage in the other.

A wide range of investigations were done, the results of some of the most significant being: a serum bilirubin of 4–32 mg./100 ml., about 60% of which was conjugated; a grossly abnormal bromsulphthalein test; raised alkaline phosphatase; transaminases at first normal but in the later stages of the disease abnormal; steatorrhoea; bilirubinuria and increased urobilinogen in the urine.

No pigment was found in the liver biopsy, which was essentially normal, and no extrahepatic obstruction was found at laparotomy. Bile acids were estimated before and after treatment with cholestyramine. The effect of this bile acid sequestering agent is given.

The results on these patients suggest a new defect in bilirubin transport which may be an inborn error of metabolism. Its relationship to similar cases described by Rotor and others is discussed.

**15. Liver mitochondria as a source of plasma enzymes.** Max M. Friedman (*Chemistry Division and the Dr. Milton J. Goodfriend Laboratory, Department of Laboratories, Bronx-Lebanon Hospital Center, Bronx 57, N. Y.*).

Some plasma enzyme activities are known to increase in diseases associated with infarction and necrosis. These elevations have been especially noted in liver necrosis. Enzymes elevated in the plasma in liver injury are usually those systems present in high activities in the normal liver cell. The supposition has therefore been made that the plasma activity is derived from the necrotized liver cell.

Experiments in our laboratory have demonstrated that glutamic oxalacetic transaminase and fumarase do not decrease in homogenates of rat livers from animals that have received injections of carbon tetrachloride even though the plasma activities are markedly elevated. This again raises the problem as to the source of the plasma enzyme activity.

Enzyme systems in the mitochondria may be a possible source of the activity. There is evidence that the mitochondria are disrupted in liver injury resulting from carbon tetrachloride; the enzyme systems may then be "liberated" into the blood stream. This would necessitate a mechanism of transport for these complex kinetic systems from the necrotized cell to the plasma. Mitochondrial preparations disrupted by physical means, such as ultrasonic energy or hypotonic swelling, show significant increases in some enzyme activities, especially transaminase and glycolytic enzymes of the citric acid cycle. Most of the activity of these enzymes does not become apparent until the mitochondrial structure is disrupted. These findings are discussed in relation to a possible mechanism for the increased plasma enzyme activity that is seen in liver injury.

**16. A comparison of the serum leucine amino peptidase with the Hepatoscan in the investigation of suspected liver metastases.** J. B. Derrick, F. C. Heagy, R. Urtasun, and D. P. Swartz. (*Departments of Medical Research, Obstetrics & Gynecology and Therapeutic Radiology, University of Western Ontario, and the Ontario Cancer Foundation London Clinic, Victoria Hospital, London, Ont., Canada*).

The serum concentration of leucine amino peptidase (L.A.P.) is elevated in pregnancy and in diseases involving the liver, bile ducts, and pancreas. It has been observed that in most patients with hepatic metastases the serum L.A.P. is increased, while in patients with malignant disease without involvement of the liver, biliary tract, or pancreas it is within the normal range. It was thought that this relatively simple assay might be of value in the management and prognosis of the patient with malignant disease, particularly in situations where techniques such as the hepatoscan are not available. The serum L.A.P. has been estimated in 48 patients with malignant disease who were investigated also by means of scintillation scanning of the liver using radioactive Rose Bengal. The

data indicate that the two tests are of comparable value in investigating the incidence of hepatic metastases.

**17. N-acetyl-β-glucosaminidase activity of the serum and liver in hepatic damage.** Masahisa Wada and Natsuki Mitsutani (*University Hospital, First Department of Internal Medicine, Osaka University, Osaka, Japan*).

There is an increase in the content of acid mucopolysaccharides (AMPS) in the laying down of connective tissue. The observation that AMPS precipitate collagen fibrils from collagen solution seems to show the importance of AMPS in fibrogenesis. Recently, it was suggested that N-acetyl-β-glucosaminidase (NAGL) participates in the metabolism of AMPS. The purpose of the present study was to study the relationship between NAGL activity and AMPS metabolism in fibrogenesis of the liver, both experimentally and in human subjects.

In 15 healthy adults, NAGL activity was 7.7 ± 1.2 U. No marked sex difference was seen. Marked elevation of serum NAGL activity was observed in patients with various hepatic diseases. The highest activities of serum NAGL were observed in patients with chronic hepatitis (17 cases) and hepatic cancer (12 cases). Serum NAGL activity was significantly higher in early cirrhosis of the liver when compared with the serum of advanced cirrhosis. Changes in NAGL activity of the serum paralleled the urinary excretion of AMPS in patients with chronic hepatitis and cirrhosis of the liver.

The NAGL activity both of the serum and liver of rats with experimental liver damage produced by carbon tetrachloride inhalation increased gradually, reaching the highest value in the third month and then decreasing slightly in the fourth month. The concentration of AMPS in the liver of these animals showed similar findings.

These findings seem to support the idea that serum NAGL activity reflects the abnormalities in the metabolism of AMPS of the liver during the process of hepatic fibrosis.

**18. Serum adenosine deaminase in the jaundiced patient.** D. M. Goldberg (*Department of Biochemistry, Western Infirmary, Glasgow, Scotland, U. K.*).

Serum adenosine deaminase (A.D.) was estimated by a spectrophotometric technic adapted from the method of Solomon in over 200 cases which fell into one of four groups:

A. Normal: Blood donors and patients admitted for elective operations in whom no organic illness was demonstrated or suspected

B. Patients with established disease such as carcinoma and congestive cardiac failure in whom hepatic complications were suspected clinically, but disproved by conventional laboratory tests and by postmortem examination in those who succumbed

C. Patients with obstructive jaundice

D. Patients with viral hepatitis, drug hepatitis, and portal cirrhosis who were clinically jaundiced at time of examination

With few exceptions, the patients in Group B fell within the same range of activity as those in Group A. Of those patients in Group C, approximately half fell within the normal range and half exceeded it. All the patients in Group D gave values outside the normal range; the overlap between Groups C and D did not exceed 10% of each.

Comparison of the discriminatory ability of A.D. with that of serum transaminases (S.G.O.T. and S.G.P.T.) and ornithine carbamyl transferase (O.C.T.) revealed that A.D. was raised as frequently as the others in acute hepatocellular disease, though the percentage elevation was considerably less, while in chronic hepatocellular disease, A.D. was raised more frequently than O.C.T. and as frequently as the transaminases. In obstructive jaundice, A.D. was raised less frequently than the transaminases and as infrequently as O.C.T.

It is concluded that the assay of serum A.D. is a useful adjunct to existing procedures for distinguishing between chronic liver damage and biliary obstruction in the jaundiced patient.

**19. Histidase and urocaninase of the blood in liver lesions.** S. R. Mardashev (*Department of Biochemistry, The First Moscow Medical Institute, Moscow, USSR*).

As histidase (histidinedeaminase) and urocaninase are found only in the liver, it was interesting to study the possible appearance of these enzymes in the blood during various lesions of the liver.

Experiments carried out on rats in our laboratory demonstrated that poisoning of rats with carbon tetrachloride, which led to necrotic destruction of the liver, proved that it is always possible to detect histidase and urocaninase activity in the blood serum.

Our further observations showed that there is no histidase and urocaninase activity in the blood serum in people who are considered fairly healthy. Nevertheless one may detect marked histidase and urocaninase activity in the blood serum of people suffering from virus hepatitis.

**20. A rapid modification of the cephalin cholesterol flocculation test.** Arthur A. Rosenberg, Lucille G. Eimann, and John O'Leary (*Laboratory Service, Veterans Administration Hospital, Albany, N. Y.*).

The standard cephalin cholesterol flocculation test has been modified so that results are available in 5 hr. Test constituents and volumes remain as described in the original technic by Hanger. The cephalin cholesterol antigen-serum mixture is incubated in the refrigerator for 4 hr., then centrifuged. The supernate is discarded, and sedimented antigen particles are resuspended in fresh saline.

By turbidimetric comparison of the individual specimen suspension with a suspension control, the degree of antigen particle flocculation is determined and converted to the customary reportable plus values.

Interference of bilirubin and chylousness because of lipemia is markedly diminished since the supernate is discarded. Refrigeration is generally preferable over the 37° incubation suggested by Bunch to elicit maximum flocculation in the incubation period. Utilization of the colorimeter reduces the subjective element of interpretation common to the standard procedure. Test results from a large number of positive, borderline, and negative specimens in both the rapid and standard procedures compare favorably.

**21. Qualitative changes of human plasma proteins in cirrhosis of the liver.** T. R. Niederland (*III. Medical Clinic and Research Laboratory of Pharmacobiochemistry, Komensky University Medical School, Bratislava, Czechoslovakia*).

The salicylemic level in the blood after I.V. injection of sodium salicylate is lower in patients with liver cirrhosis than in healthy individuals and in patients with other diseases.

In order to clarify the mechanism of this test the excretion of salicylate metabolites (free salicylic acid, salicyluric acid, and both salicylglucuronic acids) in the urine of patients with cirrhosis were determined by means of chromatography. No significant differences could be found when compared with the excretion in healthy individuals. The lower salicylemic levels in liver cirrhosis could not be explained by changes in salicylate metabolite excretion. Therefore the adsorption of salicylates on the plasma proteins was determined. Compared with the findings in healthy individuals, there was a decrease of adsorption capacity of plasma proteins in liver cirrhosis. The adsorption capacity of plasma albumin increases after dialysis, but remains still lower than in healthy individuals.

## *SECTION D: ELECTROLYTES*

**22. Normal ranges of acid-base control.** John M. Harrelson and Ralph E. Thiers (*Clinical Chemistry Laboratory, Duke University Medical Center, Durham, N. C.*)

Homeostasis maintains the body at a normal *p*H by means of a two-dimensional system of acid-base control. The *p*H and carbon dioxide content are not the natural axes of this control system. However a "normal metabolic control line" and "normal respiratory control line" can be defined on a diagram of *p*H versus $CO_2$. These two lines do form the physiological axes important in acid-base con-

trol and they can be used diagnostically to separate into its metabolic and respiratory components any abnormality involving acid-base derangement.

The variation from individual to individual of the positions of these two normal control lines on a diagram of *p*H and $CO_2$ content (similar to the *p*H-bicarbonate diagram of Davenport) can be considered as the normal range for metabolic factors of acid-base balance, separate from respiratory factors, and vice versa. In-vivo experimentation with normal humans and tonometry experiments on samples of human venous blood have been employed to determine these normal ranges. The methods used and the results obtained are discussed, along with the diagnostic utilization of the data.

**23. Quantitative aspects of acid-base balance.** Ralph E. Thiers and John M. Harrelson (*Clinical Chemistry Laboratory, Duke University Medical Center, Durham, N. C.*).

Several systems are currently employed for determining acid-base status. The approach involving metabolic control lines and respiratory control lines on a *p*H-$CO_2$ content chart has proved conceptually simple and qualitatively accurate in diagnostic application. However, therapy demands a quantitative estimate of acid or base excess. There is some divergence in the approaches of Singer and Hastings, Astrup, and Davenport. In terms of technics of both measurement and interpretation the systems employed are complex. A simplified method of quantitative estimation of the acid-base status of patients is presented and the differences between the various approaches discussed.

**24. Ultramicrotechnic for the determination of total bases of plasma.** F. Teixeira, A. Martins, F. Moura Preza (*Laboratory of the Department of Pediatrics, Lisbon University Faculty of Medicine, Lisbon, Portugal*).

An ultramicrotechnic for the determination of total cations in biological fluids is described. The method, similar to that of Natelson, consists of the electrodialysis of a 20:1 aqueous solution of the liquid to be studied. Into this solution, which is contained in a tube connected by a platinum electrode to the +pole, is placed a second tube, closed with a cellophane membrane on which is placed 0.1 ml. Hg and 0.2 ml. 2% boric acid. The other connection is made by means of a platinum electrode connected to the Hg drop (—pole). The current is furnished by an apparatus utilizing the following: (1) an auto transformer for connection to 110 or 220 v. lines; (2) a full-wave selenium rectifier which reduces the ripple voltage and improves the output current; (3) an adjustable resistance that limits the initial electrolysis current, being switched off a few minutes after; (4) an output D.C. voltmeter that controls the voltage applied to the electrodes; (5) a D.C. ammeter that gives permanent indication of actual electrolysis cur-

rent and permits plotting curves of current versus time; and (6) a pilot bulb, which, when lit, indicates a switched-on instrument (this is optional).

The dialysis is completed in 15 min. The titration of the cations combined with the mercury is made directly with standard HCl (ultramicrotitrator).

**25. Practical suggestions for the determination of sodium and potassium in serum.** Jonas Bergström, Eric Hultman, and Bertil Josephson (*Central Clinical Laboratory, St Erik's Hospital, Stockholm, Sweden*).

In the flame photometrical analysis of serum sodium and potassium, the presence of serum proteins may give rise to erroneously low values. This error does not seem to be adequately compensated for by the addition of surface-active substances or solutions. On the other hand, filtrates from sera precipitated by trichloracetic acids showed sodium and potassium values identical with those obtained by activation analysis. A precipitation-dilution modification is described suitable for determination of sodium and potassium in one dilution.

**26. Activation analysis of tissue electrolytes.** Jonas Bergström (*The Central Clinical Laboratory and the Renal Clinic, St. Erik's Hospital, Stockholm, Sweden*).

Sodium, potassium, chloride, and phosphorus in needle biopsy specimens from human tissues were determined by means of activation analysis. The water content was determined by weighing before and after drying. Fat was extracted with petroleum ether. The dry and fat-extracted samples were irradiated with thermal neutrons in an atomic reactor. An irradiation time of 40 min. was used for sodium and chloride determination. $Na^{24}$ and $Cl^{38}$ activities were measured with a well scintillation counter, and a single-channel pulse-height analyzer was used for the discrimination. After a few days the samples were irradiated once again, this time for 24 hr. Geiger counting with suitable aluminum filters was used for the discrimination between $Na^{24}$, $K^{42}$, and $P^{32}$.

Good agreement was found between results obtained by activation analysis and results obtained by chemical and flame-photometric methods.

The activation analytical methods described are simple, reliable, and very sensitive and, thus, well-suited for analysis of small tissue specimens.

**27. Metabolic studies in a patient with aldosterone-secreting tumor.** James C. Eaton (*University Department of Pathological Biochemistry, The Royal Infirmary, Glasgow, Scotland, U. K.*).

The metabolism of a patient with an aldosterone-secreting tumor was studied at varying levels of potassium intake and while the patient was receiving spirona-

lactone. The findings show that, in presence of the tumor, a relationship between plasma potassium and urine potassium persists, but that the effect of the tumor and of spironalactone was to disturb this relationship so that the amount of potassium excreted at any given level of plasma potassium was abnormal. Spironalactone administration and removal of the tumor each caused a fall in the previously high plasma $CO_2$.

Although the patient had accumulated 1800 mEq. of potassium before operation by intensive potassium therapy, biopsy of muscle at operation showed only ⅔ of the normal potassium content.

**28. The determination of fluoride in urine by microdiffusion.** L. Dubois, T. Teichman, and J. L. Monkman (*Department of National Health and Welfare, Occupational Health Division, Ottawa 4, Canada*).

Fluorides are of increasing significance in our present civilization in terms of the amounts processed industrially, and because of the health implications. It is necessary to be able to analyze air, biological, water, and soil samples with accuracy to determine possible exposures in the human environment.

In the past, the analysis of fluoride in urine was based on the well-known Willard and Winter distillation, which separated the fluoride from the rest of the sample. This was usually followed by a thorium nitrate titration. Considerable skill and experience has been required with this method.

A recent improvement is the use of ion-exchange separation in place of the distillation. Ion-exchange separation is quicker and more reliable. Microdiffusion has also been found to be a suitable method of separation. This procedure lends itself to large-scale analysis of urine samples with no loss in accuracy or precision.

Some comparisons are made of urinary fluoride values found by distillation, ion exchange, and microdiffusion. The accuracy and precision are evaluated and the "normal" value for fluoride in human urine is discussed.

**29. Gasometric analysis of carbon monoxide in air.** Julius Sendroy, Jr., and John D. O'Neal (*Division of Chemistry, U. S. Naval Medical Research Institute, National Naval Medical Center, Bethesda, Md.*).

Methods of Van Slyke analysis for CO in air, previously described by the senior author, have been improved in respect to flexibility of operation and procedure over an expanded range of concentrations. The technic described is especially designed for use in the toxicologically important range of 0.005-0.1% and serves as a quantitatively adequate and preferred substitute for the standard, more difficult, and time consuming iodine pentoxide method.

## *TUESDAY, AUG. 20*

### *SECTION A: COAGULATION*

**30. Kinetics and distribution of $I^{131}$-labeled rabbit anti-human-fibrinogen in man.** David Marrack, William C. Dewey, William F. Bale, and Raymond G. Rose (*Departments of Pathology, Physics, and Medicine, The University of Texas M. D. Anderson Hospital and Tumor Institute, Houston, Tex., and Department of Radiation Biology, University of Rochester School of Medicine and Dentistry, Rochester, N. Y.*).

The presence of an antigen and the corresponding homologous antibody in the circulation of an animal results in complex formation and its rapid clearance from the circulation ($t\frac{1}{2}$=5–15 hr.).

Purified $I^{131}$-labeled rabbit anti-fibrinogen ($I^{131}$-antibody) cross-reacts with fibrin. In animals and man this antibody behaves anomalously when given intravenously, having a biological half-life of about 8 days. The antigen is in excess in this system. In a proportion of the patients studied 1–2% of the $I^{131}$-antibody injected accumulates in tumor sites. This concentration represents greater than 1000 times the amount expected from the blood and extracellular fluid content of the tumor. Fibrinolysis is blocked by epsilonaminocaprotic acid (EACA).

$I^{131}$-labeled fibrinogen similarly concentrates in some malignant tumors. Its biological half-life appears to be shorter (3–5 days) than that of the $I^{131}$-antibody.

It is concluded that in some patients with malignant tumors, a significant portion of both the circulating fibrinogen molecules and fibrinogen-$I^{131}$-antibody complexes are removed from the circulation by the conversion of fibrinogen to fibrin at the tumor sites. Differences in kinetics between labeled fibrinogen and fibrinogen-antibody complexes is suggested by their different half-lives.

**31. Comparative study of technics for the isolation and estimation of plasma fibrinogen.** Jesse F. Goodwin (*General Clinical Research Center Wayne State University College of Medicine, Detroit 2, Mich.*).

In a study prompted by the need for an accurate, simple, and reproducible micromethod for the reasonably rapid estimation of plasma fibrinogen three methods were employed for separating fibrinogen from plasma: (1) heat coagulation, (2) thrombin interaction and, (3) sodium sulfite fractionation. A modified biuret method employing Benedict's qualitative glucose reagent and con-

centrated base was used for estimating the protein concentration of the isolated fibrinogen. No significant differences in protein content were noted in fibrinogen separated by these three technics. The salt fractionation technic, however, showed a greater degree of reproducibility. Turbidimetric measurements of clot density resulting from thrombin interaction with fibrinogen showed a poor correlation with protein values obtained by the salt fractionation procedure.

A comparison was made of three technics for measuring the protein content of fibrinogen obtained by sodium sulfite fractionation. The technics employed utilized (1) the phenol-biuret reaction, (2) ultraviolet spectrophotometric measurements and, (3) a modified biuret reaction. Excessive sodium sulfite seriously interfered with the spectrophotometric measurements. Results from the modified biuret and phenol-biuret technics exhibited good correlation. Although it is not quite as sensitive as the other two technics, the modified biuret technic afforded a greater degree of reproducibility for estimating fibrinogen.

Plasma fibrinogen values were obtained from 94 normal individuals representing an age range of 3 to 59 years. These values are discussed in addition to fibrinogen levels obtained from a group of 80 persons with rheumatoid arthritis.

**32. A dye-uptake technic for plasma fibrinogen quantitation.** J. A. Foreman, G. L. Douglas, L. M. Bergquist, and R. L. Searcy (*Departments of Pathology, Los Angeles County General Hospital (Unit 2) and California College of Medicine, Los Angeles, Calif.*).

Acute cases of hemorrhage frequently necessitate rapid evaluation of plasma fibrinogen levels. In order to facilitate these measurements, a technic has been devised whereby fibrinogen concentrations are quantitated in terms of Ponceau S uptake.

Aliquots of 0.2 ml. of plasma are added to 6.0 ml. quantities of buffered diluent containing 0.04% Ponceau S. Fibrinogen is converted to fibrin by addition of 50 U. of topical thrombin dissolved in glycerol-water (1:1). After allowing 15 min. for clot formation, fibrin is sedimented by several minutes centrifugation. The supernatant is decanted and the stained fibrin is treated with approximately 10 ml. of 5% acetic acid to remove free dye. Ponceau S bound to fibrin is then extracted into 6.0 ml. of 0.1 N sodium hydroxide. Amounts of dye recovered are quantitated spectrophotometrically and converted into fibrinogen concentrations.

After evaluating a number of possible technical variables, the proposed method was applied to a series of normal and pathologic plasmas. Fibrinogen values yielded by the Ponceau S procedure agreed favorably with those obtained by the biuret color reaction. The dye technic is rapid, reliable, and appears suitable for most clinical and research purposes.

**33. Blood lipids in relation to coagulation.** N. F. Maclagan and J. D. Billimoria (*Department of Chemical Pathology, Westminster Medical School, London, S.W.1. England*).

Blood lipids from normals and from patients with ischemic heart disease (I.H.D.) have been compared. Stypven clotting times, lipemia, and levels of total lipids, triglycerides, esterified and free cholesterol, and phospholipids were examined. The phospholipids were further fractionated into cephalins, lecithins, sphingomyelins, cerebrosides, and lysolecithins. Stypven coagulation activities of each of these fractions showed that the activity from plasma, platelets, and red cells were confined to the cephalin group—mainly phosphatidylethanolamine (P.E.).

Except for small increases in free cholesterol and sphingomyelins in I.H.D., no major differences in lipid levels were observed. However the Stypven clotting activity of the I.H.D. cephalin was 70 U./mg. compared with only 5 U./mg. for the normal cephalin. Fatty acid analysis by gas chromatography showed a significantly higher content of oleyl cephalin in the I.H.D. group.

In-vitro experiments indicated that distearyl or dipalmityl P.E. had negligible clotting activity (1–3 U./mg.), whereas dioleyl P.E. (100 U./mg.) was strongly active. Human platelet cephalin preparations containing a high percentage of oleic acid had similar clotting activities (65–200 U./mg. of cephalin). It is inferred that oleyl P.E. is the lipid component of blood which is responsible for its activity in the stypven system.

**34. Standardization of the one-stage "prothrombin time" assay.** Alan Mather (*The Memorial Hospital, Wilmington, Del.*).

Reliability of single-stage assays can be greatly improved by standardization of technic by mechanical clot-timing and by evaluation of the adequacy of thromboplastin reagent by correlating its performance with a theoretical relation between plasma thrombin generating activity (TGA) and clotting time.

Ordinarily this relation is assumed to approximate a straightforward hyperbolic reaction of second order in which the "dilution curve"—usually in adsorbed plasma—approaches linearity in a simple plot of log concentration vs. log time. While this relationship holds for preformed thrombin, the generation of triggering levels of thrombin in plasma after addition of thromboplastin requires a minimal "generation time," $a$, to which the curve becomes asymptotic at infinite concentration. With adequate thromboplastin and a standardized mechanical assay technic, the relation between clotting time and TGA can be predicted for saline dilutions of plasma; a linear plot is obtained with log $c$ vs. log $(t - a)$, or

more simply, dilution ($1/c$) vs. time. The relation follows the equation given by Quick, but almost universally ignored:

$$c = \frac{k}{t - a}$$

The value of $a$ (8–11 sec.) is dependent upon anticoagulant, reagent, and technic employed, but apparently is independent of concentration of reactants; $k$, the rate constant, is also dependent upon reagent and test conditions but its numerical value (3–4) is determined by the reference "normal" concentration.

By frozen storage of large calibrated lots of lyophilized reagent and of citrated pooled plasma control, a single calibration curve can be maintained for the assay.

**35. Laboratory control of anticoagulant therapy.** S. Winsten (*Department of Laboratories, Albert Einstein Medical Center, Philadelphia 41, Pa.*).

In many clinical chemistry laboratories throughout the country, the prothrombin time determination has become a significant portion of the routine laboratory work load. It has become necessary, therefore, not only to institute rigid laboratory control methods but also to automate at least part of the procedure. This report summarizes 18 months experience with a new prothrombin timer, the EEL prothrombin meter (Evans Electroselenium Co., Ltd., Halstead, Essex, England). The principle of the operation of this instrument is somewhat different from presently available commercial machines. Its major component is a photoelectric circuit that is sensitive to optical density changes ocurring during the formation of the fibrin clot. At the completion of the reaction a synchroclock automatically shuts off and the prothrombin time is read in seconds on a dial on the face of the instrument.

When potassium oxalate is used as the anticoagulant in prothrombin tubes, calcium is removed by precipitating as calcium oxalate. This precipitate interferes with the blank setting. It is necessary then to have some other anticoagulant in the tubes collected for prothrombin time estimations on this prothrombin timer. Before using the EEL prothrombin meter in the routine laboratory the role of tube anticoagulants in prothrombin time determinations was investigated. The results of the studies are reported and information is presented which indicates that citrate is a more satisfactory tube anticoagulant than oxalate.

### *ATOMIC ABSORPTION*

**36. Estimation of magnesium by atomic absorption spectrophotometry.** D. B. Horn and A. L. Latner (*Department of Clinical Chemistry, The Medical School and Royal Victoria Infirmary, Newcastle-upon-Tyne, England*).

Using a Hilger atomic absorption spectrophotometer attachment and an Optica spectrophotometer, excellent results have been obtained in relation to the deter-

mination of magnesium in biological fluids, including blood and urine. The calibration curve has proved remarkably reproducible and recoveries of added magnesium have been excellent both from the original biological fluids themselves and from such fluids rendered magnesium-free by ion-exchange resins. An account is given of the factors necessary to ensure results of high standard with particular reference to the delivery rates of propane and air; the method is very sensitive to changes in these. An account is given of the results obtained in normal individuals and in disease states.

**37. The determination of serum calcium using atomic absorption spectrophotometry.** A. Zettner and D. Seligson (*Grace-New Haven Community Hospital and Yale University School of Medicine, New Haven 4, Conn.*).

A new flame photometry technic based on the principle of atomic absorption was applied to the determination of calcium in serum and other biological fluids. While the technic offers most of the conveniences of emission flame photometry, it did not suffer from spectral interferences caused by serum constituents. Chemical and physical interferences were caused by phosphates, sulfates, and serum proteins, all of which depressed absorption. The sensitivity of the method permits a 1:10 to 1:20 dilution of serum samples for routine analysis. A determination requires 0.1 to 0.3 ml. of serum. A diluent was developed which was capable of abolishing interferences and made possible measurement of Ca in directly diluted serum samples without prior removal of any serum constituents. The coefficient of variation between replicates was less than 1%. Generally good agreement was found between values obtained by the atomic absorption technic and the oxalate-permanganate method, although atomic absorption results tended to be lower by 3%. A detailed study of specificity indicated the atomic absorption method was correct.

This atomic absorption method appears to be superior to existing conventional methods for serum calcium in regard to accuracy, precision, and ease and speed of performance. It is recommended for use in service and research laboratories as the method of choice for calcium.

**38. An application of atomic absorption spectrometry in clinical chemistry: Determination of copper in biological materials.** Eleanor Berman (*Pathology Department, Illinois Masonic Hospital, Chicago 14, Ill.*).

Atomic absorption spectrometric technics are incorporated with a modified chemical method in the determination of copper contents of blood, urine, and tissues. Copper is removed from acid-digested samples by chelation with EDTA and diethylthiocarbamate and extraction with a ketone. The extracts are read in an atomic absorption spectrometer (Perkin-Elmer Corp.) against copper standards similarly treated.

Copper levels found in blood and urine of random hospital patients apparent-

ly free from neurological disorders and levels found in patients with neurological problems are compared.

**39. Determination of trace metals in biological materials by atomic absorption spectrophotometry.** Walter Slavin (*Perkin-Elmer Corporation, Norwalk, Conn.*).

Atomic absorption spectrophotometry has proved to be a very convenient means to determine metals in various biological media. Methods have already been described to determine zinc and cadmium at their natural level in urine without sample preparation. Other metals have been determined in urine with simple extraction methods. The determination of cadmium, chromium, thallium, lead, and cobalt in blood and urine at toxicological levels without pretreatment of the sample is discussed. Also discussed is the determination of a number of other important trace metals at their natural levels in body fluids by methods that require a minimum of sample pretreatment. This has become possible as a result of improvements in instrumentation that provide increased sensitivity.

## *SECTION B: QUALITY CONTROL*

**40. Biological versus analytical errors.** Robert P. Harpur (*Institute of Parasitology, McGill University, Macdonald College Ste. Anne de Bellevue, P.Q., Canada*).

Chemists traditionally analyze in duplicate, but statisticians have suggested that this may not always be the most efficient procedure. This suggestion is examined as it applies to biochemical analyses. An example is the analysis for carbohydrate of a biological material (ascaris muscle) in which the carbohydrate is made up almost entirely of glycogen and trehalose. The variation in the determinations is broken down into biological error, error in precipitation or extraction, and error in colorimetric determination. With glycogen the biological error is far greater than the analytical error and repeated samples rather than repeated analyses gives the greatest increase in precision of the analyses. With trehalose the biological error is of the same order of magnitude as the analytical error. In this case improvement in the method and duplication of analyses can increase the over-all precision.

**41. Quality control in clinical chemistry: I. A description of an intermedical laboratory quality-analysis survey.** C. J. Porter. (*Department of Laboratories, Toronto General Hospital, Toronto, Canada*).

In 1962 over a 10-week period 12 medical laboratories in Toronto and vicinity carried out a cooperative study to obtain information pertaining to the precision and accuracy of 10 routine chemical tests and of 3 routine hematological tests.

The 10 chemical procedures examined were for urea, glucose, Ca, Cl, creatinine, P, K, Na, total protein and A/G ratio. The three hematological procedures considered were hemoglobin, red blood count, and hematocrit. In carrying out the chemical study two dilutions selected at random from seven possible dilutions of a commercially available reference preparation were analyzed for most of the blood constituents under study by each of the participating laboratories on each working day from Apr. 16 to June 29, 1962. The hematological determinations were carried out each day at two dilution levels on blood obtained from a patient on whom, on the day in question, a hemoglobin determination had been requested by the patient's physician. In preparing specimens for hematological examinations, ABO plasma was employed as diluent.

The results presented include the mean values of all tests and the within-day variation, the between-day variation, and the over-all variation of single determinations for each procedure studied in each of the participating laboratories.

Discussions among the laboratory personnel who participated in the survey brought to light information about certain of the factors responsible for the observed variations in the results obtained. Some of the conclusions arrived at during the postsurvey analyses are presented.

**42. Quality control in clinical chemistry: II. Application of information obtained from an intermedical quality analysis survey.** D. M. Young (*Department of Laboratories, Toronto General Hospital, Toronto, Canada*).

The results obtained from the Intermedical Laboratory Quality Analysis Survey (Abstract 41) have been reviewed in joint meetings of members of the laboratory and clinical staffs of the participating institutions. An account is given of certain of the impressions gained relating to: (1) the clinical significance of the variations observed; (2) the degrees of accuracy and precision which, in the present state of medical knowledge, ought to be aimed for by the routine clinical laboratory; and (3) the place and purpose of quality analysis and of quality control in the clinical laboratory.

The general conclusions offered are:

1. For most of the procedures under examination (e.g., glucose, B.U.N., Hb., etc.) so far as the laboratories surveyed are concerned at the present time the imprecision of the clinical information available is much more important than lack of precision in the laboratory in limiting the practical usefulness of laboratory information.

2. In a few situations (e.g., serum calcium and creatinine in clearance studies) none of the laboratories involved is adequately meeting the clinical need.

3. So far as the laboratories which participated in the survey are concerned, quality control would seem to have its greatest immediate practical usefulness in programs designed to extend knowledge relating to the "concentration ranges" and the "concentration profiles" of body constituents in clinically defined states of health and disease.

**43. The reliability of clinical chemistry determinations: Computer analysis of day-to-day variability.** Martin H. Murray and Anthony J. LaPaglia (*Departments of Chemistry, Genesee Hospital and Highland Hospital, Rochester, N. Y.*).

A joint quality control program of two laboratories used one lot each of six different types of commercially available control sera. Determinations were performed each working day in each hospital using the same lot prepared by one individual. The lots were varied by a random system to avoid familiarizing the operators with the values. In the course of a year some 9000 items of data were accumulated.

Computer-implemented statistical analysis of these data provides information on the day-to-day variability of clinical chemistry procedures. It also provides comparisons between laboratories, between procedures, and between manual and automated methods.

The coefficients of variation of the methods illustrate the increase in precision attained by improved instrumentation and simplification of procedures. The same parameter serves as a "reliability index" of the methods.

**44. Evaluation of serum preparations used for controlling total cholesterol measurements** L. M. Bergquist, J. A. Giddings, and R. L. Searcy (*Departments of Pathology, Los Angeles County General Hospital (Unit 2) and California College of Medicine, Los Angeles, Calif.*).

Interlaboratory variations with respect to results of serum cholesterol measurements point to the need for more rigid control of these analyses. Unfortunately, no completely satisfactory standardization criteria have yet been established. A spectrum of serum preparations has been proposed to enhance the accuracy of cholesterol determinations. However, many of these materials have not yet been extensively evaluated. For this reason, a preliminary study was made of a number of commercially available serum preparations with assigned cholesterol concentrations. Stability and uniformity of dried as well as reconstituted products were assessed under various conditions. Recoveries of cholesterol were estimated in terms of three accepted technics which entail different color reactions. Immunochemical and electrophoretic properties of the products were compared and found to differ. These investigations suggest that routine use of particular preparations under standardized conditions may significantly increase the reliability of serum total cholesterol estimations.

**45. The use of probability paper in analyzing frequency distributions.** D. H. Curnow and Keith Sheard (*Royal Perth Hospital and University of Western Australia, Perth, Western Australia*).

The use of probability graph paper in resolving polymodal frequency distributions was described by Harding and extended by Cassie.

The cumulative percentage frequency distribution is calculated from the raw data and plotted on the probability paper. A straight line indicates a normal distribution and a curve indicates departure of one sort or another from normal. A curve with inflexion points indicates a composite distribution. A composite distribution may be resolved by identifying the inflexion points, isolating the relevant part of the curve, transforming it to 100%, and replotting the result. The mean and standard deviation of the whole curve, or of any part so transformed can be read directly from the paper. It is possible with this simple graphical method to make preliminary examinations of clinical data and to separate various possible components of composite statistical populations. Significance may be tested by the chi-square method, providing smoothed data are not used.

The method is used to examine the statistical distribution of 1000 cases where total protein and glucose in cerebrospinal fluid were measured. Several statistical populations are present in these distributions and there is a sex difference in protein but not in glucose values.

The distribution of 2000 blood urea values is also examined. The distribution is slightly skewed, the skewness being due to the presence of three overlapping populations. In the case of the major population, approximately 75% of the sample, the distribution was normal, with a mean of 29 and standard deviation of 8 mg./100 ml. for both sexes.

## *SECTION C: ELECTROPHORESIS AND CHROMATOGRAPHY*

**46. Effects of tetracyclines on electrophoretic mobilities of serum lipid-bearing proteins.** R. L. Searcy, J. A. Foreman, and L. M. Bergquist (*Departments of Pathology; Los Angeles County General Hospital (Unit 2) and California College of Medicine, Los Angeles, Calif.*).

Although widely used therapeutically, little information is available concerning possible interactions of tetracyclines with serum components. In order to further clarify mechanisms of tetracycline transport in vivo, small quantities of tetracycline (TC), chlortetracycline (CTC), oxytetracycline (OTC), or demethylchlortetracycline (DTC) were added to serum. Such treatment with TC or CTC produced a marked increase in serum lactescence, whereas DTC elicited a prompt precipitation reaction. By contrast, serum-OTC mixtures were not visibly altered.

Aliquots of tetracycline-treated sera were subjected to electrophoresis on filter paper and viewed in ultraviolet light. Although patterns differed, in most instances a sharp fluorescent band was clearly detectable near the point of serum application. Treatment of electropherograms with bromphenol blue disclosed that fluorescent zones corresponded to well-defined protein-staining bands. Strips prepared from TC-serum mixtures stained with Oil Red O demonstrated

that the new electrophoretic zone contained all lipophilic material which normally migrates as alpha- and beta-lipoproteins.

Bromphenol blue-stained areas were eluted from electrophoretic strips prepared from serum before and after addition of TC. The decreased dye uptake of alpha- and beta-globulin fractions of TC-treated serum could be accounted for in the new electrophoretic zone containing serum total lipoproteins. Therefore, TC interactions with serum make possible simultaneous estimation of serum protein and lipoprotein levels on single electrophoretic strips.

**47. Electrophoresis in synthetic gel: II. Molecular vs. gel factors.** Samuel Raymond, Masumi Nakamichi, and Ina Frank (*Pepper Laboratory of Clinical Medicine, School of Medicine, University of Pennsylvania, Phila. 4, Pa.*).

Classical electrophoresis theory, in which electrophoretic migration is directly proportional to field strength and time at constant temperature and *p*H, is inadequate to account for electrophoretic migration through a gel medium. Three groups of factors affecting migration rate can be distinguished: (1) molecular factors such as diffusion constant, adsorption, size, and charge; (2) gel factors such as porosity, ion-exchange capacity, and electrosmosis; and (3) buffer factors such as chemical composition, concentration, and conductivity. Independent measurement of these factors individually provides data for testing the theory and also affords a basis for rational selection of experimental conditions to obtain optimum resolution.

Application of this viewpoint to experimental results using normal serums and various test mixtures still leaves a residue of unexplained effects apparently related to age of gel, preconditioning, and perhaps condition of gel surface. Two-dimensional electrophoresis affords a convenient experimental technic for the study of these effects.

**48. Electrophoretic separation of RNA nucleotides on agar gel-paper.** B. Zak and L. M. Weiner (*Departments of Pathology and Microbiology, Wayne State University College of Medicine, Detroit, Mich.*).

Low concentrations of agar may be used to make up a gel in a buffer system appropriate for the electrophoretic separation of the RNA nucleotides, cytidylic, uridylic, adenylic, and guanylic acids. When the gel overlays vegetable parchment paper, a convenient technic for the subsequent handling of the dried and separated nucleotides becomes available. These rapidly moving and easily separable compounds can be eluted with dilute alkali for qualitative identification and quantitative determination.

Some of the details of the procedure described include:

1. Rapid electrophoretic separation of these model nucleotides in an uncovered gel overlaying vegetable parchment paper
2. Ultraviolet scanning of the eluates of the separated nucleotides

3. The use of the difference spectra in the ultraviolet range for a qualitative picture of the nucleotides.

**49. A rapid quantitative method for concentrating urine for protein electrophoresis.** Hylton McFarlane (*University of the West Indies, Department of Pathology, Kingston 7, Jamaica*).

Several methods have been reported in the literature for concentrating urine for protein electrophoresis. Unfortunately, each of these methods suffers from one defect or another. The present paper describes a method which is simple, rapid, and can be readily quantitated.

Urines from hospitalized patients were dialyzed against sucrose for approximately 2 hr. Electrophoresis was then carried out on cellulose acetate strips. For comparison the serum of each case studied was run on the same strip along with the urine.

The total protein of the concentrated urine was estimated on the AutoAnalyzer, whereas the albumin and globulins were calculated from the scanned electropherogram. The entire procedure required 3½ hr. and several specimens of urine could be concentrated simultaneously.

No extra equipment is necessary and the procedure can be performed in a routine clinical laboratory.

**50. A new method of recording the light absorption of the eluate of a chromatography column.** Denis Boutin and Eleanor Harpur (*Laboratories, Hôpital St. Joseph, LaTuque, Québec, and Department of Biochemistry, The Montreal Children's Hospital, Montreal, Canada*).

The manual recording of the light absorption of a large number of samples coming from a chromatography column is a difficult task requiring a great deal of time.

A continuous-flow quartz cell was developed adapted to the Beckmann D.B. spectrophotometer. The light absorption of each sample is recorded for 10 sec. This permits not only a reduction in the length of recording paper but also allows one to see exactly the shape of the curve of the eluate. Using this system with an appropriate wave length the curve of light absorption at the moment of elution from the column can be obtained.

**51. Urea, a new stain in organic acids paper chromatography.** M. J. Halpern (*Department of Physiological Chemistry, Faculty of Medicine, Lisbon, Portugal*).

The utilization of urea as a reagent to characterize citric acid in spot tests has been described by Feigl. By heating urea, ammonia and biuret are produced; ammonia reacts with citric acid forming ammonium citrazinate.

This reaction was adapted to paper chromatography and applied as well to other acids. The chromatograms are dipped in a 1% solution of urea, dried at room temperature and heated 15 min. to 140°. This reaction is very sensitive to the following acids (in U.V. light): citric, malic, transaconitic, lactic, phosphoric (sensitivity $< 1$ $\mu$g.), fumaric, glyoxylic, glucuronic, quinic, tartaric, azotic (5 $\mu$g.), $\beta$-hydroxybutyric, pyrrolidonecarboxylic, hydrochloric (10 $\mu$g.), ethylmalonic, methylmalonic (20 $\mu$g.), hypuric (30 $\mu$g.), and itaconic (40 $\mu$g.). Some acids stain in visible light, generally in higher concentrations. This reaction is not very sensitive to succinic acid (sensitivity 200 $\mu$g. in U.V. and visible light) and oxalic acid (350 $\mu$g. in U.V. light).

**52. Chromatographic studies of the carbohydrate components of the human gastric mucopolysaccharides.** J. Schrager (*Department of Pathology, Royal Albert Edward Infirmary, Wigan, England*).

Attempts have been made to study the carbohydrate components of human gastric mucopolysaccharides chromatographically. The procedure of the fractional aspirations of the gastric secretion has previously been described.

The pooled gastric secretion was treated with diastase at 37° for 5 hr., followed by dialysis at 4° for 8 days. The dialyzed secretion was concentrated under reduced pressure at 30° to a minimum of its volume and hydrolyzed using Dowex 50 as a catalyst. The sugars were developed by the descending method using three redevelopments.

Results do not support the well-established assumption that the gastric mucopolysaccharides contain uronic acid. The chromatograms of 90 gastric secretions investigated showed no uronic acid, but when uronic acid was added to gastric secretion and hydrolyzed it did appear on the chromatograms. A solution of 1% chondroitin sulfate, treated as described above and chromatographed also showed uronic acid. These experiments indicate that this method of hydrolysis frees uronic acid and does not destroy it.

All chromatograms which underwent three redevelopments of a period of 160 hr. invariably showed zones corresponding to glucose and mannose. The horizontal chromatograms with 6 hr. of development showed no glucose. The presence of glucose was then confirmed by treating the preparation with glucose oxidase followed by chromatography. No glucose bands were then detected.

The carbohydrate components of the gastric mucopolysaccharides of patients with carcinoma of the stomach show quantitative ratio differences from those of all other gastric secretions investigated. In the latter galactose and fucose are the predominant sugars. The secretion of the malignant gastric mucosae revealed a marked decrease in galactose and a great increase in glucose, the galactose being often reduced to a mere trace.

Preliminary chromatographic studies of salivary mucopolysaccharides show that the carbohydrate components are similar to those of the gastric mucopolysaccharides.

**53. The determination of arginase activity by means of paper chromatography.** R. P. Barschel (*University of Saskatchewan, Regina Campus, Regina, Sasketchewan, Canada*).

This method for the determination of arginase is based on the hydrolysis of arginine to form ornithine. The enzyme and substrate are incubated for a specified time in a glycine buffer containing a trace of manganous sulfate. The ornithine formed is separated from arginine and glycine by means of paper strip chromatography using an isopropyl alcohol-acetic acid-water solvent system and the chromatograms are developed with ninhydrin. Quantitative data may be obtained by scanning the paper strips with a densitometer similar to that used for quantitative paper electrophoresis studies of serum proteins. Since the volume of solution required for spotting the paper strips is small the method may be used for micro analyses. Application of the method to serum enzyme studies is discussed.

**54. A preparative technic for isolation of materials of relativity high volatility for gas chromatographic quantitation.** Richard H. Gadsden and William M. McCord (*Department of Chemistry, Medical College of South Carolina, Charleston, S. C.*).

Preparation of materials of relatively high volatility from biological media for quantitation by gas chromatographic technic is difficult. For technical reasons, it is necessary to transfer the agent(s) to be measured from an aqueous (polar) solution to an organic solvent (nonpolar) medium for injection onto the special chromatograph column. A method is proposed for separating these materials from small quantities of blood by a fractional distillation technic incorporating a water-immiscible "carrier" type of system for their isolation. Dehydration of the isolated sample is described. The isolation, detection, and quantitative measurement may usually be accomplished within 30 min. Accuracy, based upon experiments in reproducibility and recoveries, is good. The volume of sample is small depending upon the concentration of the material being measured and the detector sensitivity to the agent. Discussion includes some anesthetic agents (methoxyflurane, Fluothane and Fluoromar), and alcohols.

## *SECTION D: HORMONES*

**55. Fractionation of urinary corticosteroids in healthy individuals and in patients with liver disease.** Inge Dyrenfurth and Lionel E. McLeod (*Department of Medicine, University of Alberta Hospital, Edmonton, Canada*).

Since many steroid hormone conversions are normally carried out by the liver, a considerably abnormal metabolism of these hormones has been observed by various technics in patients with diseases of the liver. This study describes the

quantitative measurement of the individual metabolites actually excreted in the urine of 12 such patients as compared to that of 6 healthy individuals.

Urine specimens were extracted for corticosteroids following treatment with β-glucuronidase. The extracts were separated into 40 individual components by a further modification of a paper chromatographic technic previously described (*J. Clin. Endocrinol. Metab. 18*:391, 1958). Two types of scanning patterns of the chromatograms were obtained and quantitation of the eluted materials was achieved with three color reactions. Among the steroids measured were: cortisol (F), cortisone (E), dihydrocortisol (DHF), dihydrocortisone (DHE), tetrahydrocortisol (THF), tetrahydrocortisone (THE), tetrahydroaldosterone (THAl), 6-hydroxycortisol (6-OHF), 6-hydroxycortisone (6-OHE), 11-desoxycortisol (S), tetrahydro-11-desoxycortisol (THS), corticosterone (B), and its metabolites (THB and THA). Also measured was a number of unidentified substances.

Most of the metabolites were found diminished in the patients. This was particularly pronounced for compounds THF, THE, 6-OHF, 6-OHE, and DHF. Compound DHE was generally normal and E sometimes greatly increased. THAl was consistently elevated, though only one of the patients presented ascites. Interpretation of the results with regard to severity of the disease, handling of administered cortisol, and action of ACTH is attempted.

**56. Evaluation of procedures for urinary 17-hydroxycorticosteroids.** W. Ragland Brown (*Bio-Assay Laboratory, Dallas, Tex.*).

To settle upon a procedure which possesses the simplicity, accuracy, and reproducibility necessary for a reference laboratory, the procedures in common use were examined and compared. Modifications with regard to solvents and technics of extraction were tried. Determination by colorimetric and fluorimetric technics were evaluated on the various procedures and modifications. Results show comparison of procedures, both with the concentrations present and with recoveries of added quantities.

**57. The determination of urinary estrogens.** David J. Watson, Joe M. Burkey, and Paige K. Besch (*Steroid Research Laboratories, Department of Obstetrics and Gynecology, Ohio State University, College of Medicine, Columbus 10, Ohio*).

A rapid and accurate method for the determination of urinary estrogens is described. This method avoids the inclusion of bulky impurities in the sample to be fractionated, contrary to other reported methods. An improved method of extraction from urine, with an intermediate alkali:methylene chloride partition avoids such contamination.

The estrogens are separated by paper chromatography and quantitated by

a modification of the Ittrich method using the Kober reaction. This method avoids sources of error such as methylation, which is difficult to achieve quantitatively on urine extracts, or spectral shifts in the final color due to impurities from the urine.

The recovery of radiolabeled estradiol to samples is much higher and shows better reproducibility than has been experienced for previously published methods.

**58. Placental transfer of a new progestin.** Paige K. Besch, Robert E. Baker, and Sibu Ghosh (*Steroid Research Laboratories, Department of Obstetrics and Gynecology, Ohio State University, College of Medicine, Columbus 10, Ohio*).

6α-methyl-17-acetoxyprogesterone (Provera) has enjoyed widespread use as a highly effective progestational agent in the recent past. It seemed of interest to determine the site of localization in maternal and fetal tissues. To this end, 500 μc. of 7α-tritium-labeled Provera plus sufficient inert material to total 40 mg. of the compound, was given by mouth 8 hr. prior to therapeutic abortion. At surgery, maternal tissues, blood, and urines were collected, as well as the fetus with attached placenta. The tissue was dissected, homogenized, and with the blood, and urine, extracted with Delsal's solution. The radioactivity (dpm) of suitable aliquots of each sample was determined in a liquid scintillation spectrometer (Packard Tri-Carb, Model 314 EX-2). Each sample was counted sufficiently long to achieve a statistical accuracy of at least ±5%. Arithmetic correction for quenching and self-absorption phenomena was made by the use of tritiated toluene additions of a known number. Very small quantities of labeled drug were recovered from the fetal tissue (range 0.01–0.1 μc.). The major portion of radioactivity in the 10 tissues undergoing study was found in the fetal adrenals, spleen, and G.I. tract. At present, the structural nature of the radioactive steroid isolated from the tissues is undergoing identification.

**59. The rapid determination of cortisol and thyroxine in plasma using gel filtration.** Beverley Pearson Murphy and Chauncey J. Pattee (*Clinical Investigation Unit, Queen Mary Veterans' Hospital, Montreal, Canada*).

Both cortisol and thyroxine are strongly bound in a highly specific manner to certain plasma proteins—viz., CBG and TBG. The binding properties of these proteins have been used to provide simple methods for the assay of these hormones.

In the determination of cortisol, 1 ml. of plasma was added to 2 ml. of ethanol, shaken, and centrifuged. One milliliter of supernatant was evaporated to dryness and 1 ml. of a standard plasma solution containing cortisol-4-$C^{14}$ was added. After shaking, this mixture was transferred to a column of Sephadex-G25. The protein-bound fraction was eluted with distilled water and assayed for radio-

activity. The amount of cortisol in the unknown sample was inversely proportional to the radioactivity and was read from a previously determined standard curve. The recovery of cortisol added to plasma was of the order of 90%. The mean 9:00 A.M. plasma level in 12 healthy subjects was 14.0 ± S.D. 4.2 μg./100 ml. For multiple determinations on a single sample, the S.D. was ± 10%.

Preliminary results in the determination of thyroxine, using a method closely similar to that of cortisol and employing thyroxine-$I^{131}$ as a tracer, gave values compatible with those of PBI determinations.

The advantages of these methods include great specificity for the hormones measured, with lack of interference by nonhormonal substances and ease and rapidity (1 hr. or less) of performance.

**60. Interference in the determination of urinary catecholamines by formaldehyde-forming drugs.** Max O. Klotz, Helmut J. Richter, and Mary T. Meuffels (*Department of Biochemistry, Ottawa Civic Hospital, Ottawa, Ont., Canada*).

Several workers have reported on the interference or noninterference of a number of drugs in the determination of urinary catecholamines by the fluorescence method with ethylenediamine. Drugs such as Mandelamine and Urotropin, which after ingestion form formaldehyde in the urine were investigated. Formaldehyde inhibits the formation of fluorescent substances from added recovery standards (arterenol, noradrenaline) on which the calculation of the catecholamines in the unknown urine is based. The degree of inhibition depends on the amount of formaldehyde present in the urine and also varies with different urines. A check for the presence of formaldehyde should be made with each urine to be analyzed for catecholamines by this method.

**61. The serotonin content of platelets in pulmonary arterial and venous blood sampled during thoracic surgery.** Neville Crawford (*Cleveland Clinic, Research Division, Cleveland 6, Ohio*).

To examine the role of the lungs in serotonin metabolism, blood samples were withdrawn during 20 thoracic surgical operations from the main common pulmonary artery and one or more of the following sites:

1. Pulmonary vein draining a "normal" lung region
2. Pulmonary vein draining a lung area involved in disease
3. Left atrium

Whole blood and platelet-rich plasma platelets were counted and the serotonin content per unit platelet determined by a spectrophotofluorometric method (Crawford, 1962). The pulmonary venous and left atrial blood values were compared with those obtained for the pulmonary arterial blood in each case. Although in passage through areas affected by disease some platelet disintegra-

tion may occur, liberating serotonin, the findings also suggest that while in transit through the lungs, platelet-serotonin release and metabolism may occur without platelet rupture. The findings are discussed in relation to the earlier studies of Toh (1956) and Giarman *et al.* (1960), who showed that lung tissue extracts contain an active principle capable of releasing serotonin from platelets and neoplastic mast cells of the mouse.

**62. The multiplicity of antibodies against insulin.** Masayasu Kitagawa and Yuichi Yamamura (*Department of Biochemistry, School of Medicine, Kyushu University, Fukuoka, and Third Department of Internal Medicine, Osaka University Hospital, Osaka, Japan*).

The multiplicity of antibodies against insulin was demonstrated by the comparison of amounts of antibodies detected with different methods and the change in antigenicity with chemical modification of insulin.

Guinea pigs were immunized with crystalline whale (sperm) insulin in complete Freund adjuvant. The immune serum was fractionated with DEAE-cellulose column chromatography and zone electrophoresis. Antibodies were localized in $\gamma$- and $\beta$-globulin fractions in the immune serum.

The amounts of antibodies were determined by the neutralization of hormonal activity of insulin (neutralizing antibody), active and passive skin tests (skin-sensitizing antibody), hemolysis of insulin-conjugated red cells (bis-diazotized-benzidine method, Stavitsky and Arquilla), and precipitation of $I^{131}$-insulin-antibody complex with anti-guinea pig $\gamma$-globulin (nonprecipitating antibody, Skom and Talmage).

The parallel relationship among the titer of antibodies was not always shown on each immune serum. Some sera showed the high titer of neutralizing and nonprecipitating antibodies and the low titer of skin-sensitizing antibody, while other sera showed reverse results.

As to the chemical modification of insulin, esterification, acetylation, diazo-coupling, iodination, and cleavage of disulfide bonds by oxidation or reduction were carried out.

In some cases, the reactivity with one antibody was completely lost, while the reactivity with other antibody was fairly retained. The A or B chain of insulin lost the activity in the active skin test and combination with neutralizing antibody, but showed the reactivity in hemolytic reaction of insulin-conjugated red cells or the passive skin test.

**63. Serum $B_{12}$ postresectionem ventriculi.** Erik Hammerich Damm (*The Central Laboratory, Skive Sygehus, Skive, Denmark*).

Two groups of patients resected (a.m. Polya) in the municipal hospital at Copenhagen are reported—83 operated upon in 1949 and 76 in 1958. Eight

years after operation 18–22% of the 1949 group had serum $B_{12}$ values below 200 $\mu$g./ml. One year after operation 21–28% of the 1958 group had values below 200 $\mu$g./ml. None showed pernicious effects in the bone marrow or in the peripheral blood. Four patients in the 1949 group had significant physical abnormalities; 2 of these patients were unable to work.

The conclusion is that (1) resection a.m. Polya does not seem to remove so much mucosa from the fundus ventriculi that serum $B_{12}$ decreases with time, or (2) the physiological pool of $B_{12}$ in the liver is sufficient to compensate for the diminished resorption of $B_{12}$.

It is recommended that serum $B_{12}$ be tested at least one year after resection and that $B_{12}$ therapy be given those whose levels fall below 200 $\mu$g./ml.

## *THURSDAY, AUG. 22*

### *SECTION A: AMINO ACID METABOLISM*

**64. Studies on isovalthine.** Shunzi Mizuhara (*Department of Biochemistry, Okayama University Medical School, Okayama, Japan*).

Isovalthine is a new amino acid isolated from the urine of hypercholesterolemic patients, such as those having myxedema, diabetes, nephrosis, and atherosclerosis. The configuration of natural isovalthine was identical with a compound synthesized from L-cysteine and (+)-α-bromoisovaleric acid. Isovaleric acid residue in the isovalthine molecule is known to be an intermediate of cholesterol biosynthesis, so the isovalthine must have some intimate relation with hypercholesterolemia.

The following results of studies on isovalthine are presented. The identification of isovalthine can be carried out by a combined method of paper electrophoresis and paper chromatography, and the determination can be best performed by an automatic amino acid analyzer.

A reproducible method for the induction of isovalthinuria has been achieved by feeding with isovaleric acid in animals such as dog, rabbit, guinea pig, and rat, which never excrete isovalthine normally. Among animals, only the cat excretes isovalthine in normal urine. Although the administration of isovaleric acid is indispensable for the induction of isovalthinuria, little added isovaleric acid-1-$C^{14}$ is incorporated into urinary isovalthine. Carbon dioxide-$C^{14}$ added together with isovaleric acid is also incorporated into urinary isovalthine in small amounts only. In these cases, urinary glutamic acid had the highest radioactivity. Acetic acid-1,2-$C^{14}$ added together with isovaleric acid, however, is incorporated to a significant extent into urinary isovalthine.

Using rat liver homogenate isovalthine can be synthesized from glutathione and isovaleric acid or α-ketoisocaproic acid, but 3-methylcrotonic acid gives

S-(1,1-dimethyl-2-carboxyethyl)-L-cysteine nonenzymatically in the same in-vitro system.

**65. The photometric determination of the tryptophan content of proteins hydrolyzed with perchloric acid.** Abraham Saifer and Shirley Gerstenfeld (*Isaac Albert Research Institute of the Jewish Chronic Disease Hospital, Brooklyn 3, N. Y.*).

The spectral curves obtained with a previously described (*Clin. Chem. 8*:237, 1962) specific colorimetric reaction for tryptophan showed absorption peaks at 560, 440, and 365 mμ. The greatest absorbance per milligram of tryptophan was obtained at 365 mμ. This more sensitive procedure was employed to study the tryptophan content of perchloric acid–hydrolyzed peptides and proteins, some of which had known amino acid composition. The advantages of using perchloric acid for the hydrolysis of peptides and proteins and the various factors which influence the tryptophan reaction are discussed.

**66. Tryptophan metabolism in patients with infantile spasms.** P. Hughes and B. D. Bower (*Children's Hospital, Birmingham, England*).

A clinical and E.E.G. study of patients with infantile spasms and oligophrenia has been made and the therapeutic value of ACTH established. The finding of Cochrane that xanthurenic acid is excreted in excess after a DL-tryptophan load of 0.4 gm./kg. body weight (0.2 gm./kg. for children over 3 years) has been confirmed. Evidence for other abnormalities in tryptophan metabolism has been sought by paper chromatography of urine after the same load of L-tryptophan only (i.e., 0.2 gm./kg.). Pyridoxine-dependent steps have been found to be rate limiting, as shown by a decrease in the excretion of 3-hydroxyanthranilic acid and increased excretion of xanthurenic acid, 3-hydroxykynurenine, and kynurenic acid. This biochemical abnormality is diminished by pyridoxine and by ACTH, but pyridoxine has been found to be of less therapeutic value than ACTH.

The storage of urine for the study of tryptophan metabolites is discussed.

**67. Tryptophan metabolism in rheumatoid arthritis.** Isobel M. Bett (*Rheumatic Diseases Unit, Northern General Hospital, Edinburgh 5, Scotland, U. K.*).

Some metabolites of the amino acid tryptophan have been measured in the urine of a group of patients suffering from rheumatoid arthritis and in a group of healthy control subjects. The most striking difference between the two groups was in the increased excretion of kynurenine and 3-hydroxykynurenine by a majority of the patients after a loading dose of L-tryptophan. Their output of

kynurenic acid and xanthurenic acid was also increased but to a lesser extent. This metabolic abnormality appeared to have no correlation with duration, activity, or severity of the disease, nor did it appear to be a result of prolonged salicylate therapy. Patients after treatment with steroids appeared to exhibit a more normal pattern of tryptophan metabolism as far as excretion of these metabolites was concerned.

The enzymes involved at this stage in tryptophan catabolism are known to be dependent on pyridoxal phosphate. Consequently the effect on tryptophan loading tests of pyridoxine, administered orally and/or intramuscularly, was studied in this group of patients. In most cases there was a dramatic fall in kynurenine and 3-hydroxykynurenine excretion.

Recently a syndrome resembling rheumatoid arthritis has been reported in a group of patients suffering from tuberculosis on treatment with isoniazid and p-aminosalicylic acid. Isoniazid is a known pyridoxine inhibitor and administration can lead to abnormality in tryptophan metabolism similar to that described above.

The results of these studies are presented and their significance discussed.

**68. A study on host-tumor relationship with special reference to amino acid nutrition and protein metabolism.** Masami Suda (*Division of Protein Metabolism, Institute for Protein Research, Osaka University, Osaka, Japan*).

Nitrogen supply from the host to the tumor tissue was examined with special reference to protein metabolism and amino acid nutrition in vivo. Evidence was obtained suggesting that tumor tissue utilized the host plasma protein rather than the amino acid supplied externally. After forced feeding of amino acid mixture, incorporation of L-phenylalanine-C(U) into the tissue protein was taken as a measure of protein synthesis in rats which were maintained on a protein-free diet for 7 days. Little difference in protein synthesis of tumor tissue was observed between the group of rats receiving a complete amino acid mixture and the other group of rats fed an incomplete amino acid mixture lacking tryptophan. The protein synthesis in regenerating liver and liver of tumor-bearing rats was decreased by about 40% on omission of tryptophan from the amino acid mixture for forced feeding. In addition, it was demonstrated in vitro that the tumor cells degrade plasma protein prior to its utilization for protein synthesis.

It was also observed, in tumor-bearing rats, that the synthesis of plasma protein was elevated to that shown by plasmapheresis of normal rats, whereas synthesis of muscle protein was depressed to that of rats maintained on a protein-free diet.

These studies support the view that the tumor tissue has a marked influence on the metabolic pattern of the host animal by making use of the host plasma for the synthesis of cancerous protein. Possible mechanisms are discussed.

**69. Detoxicating effect of L-aspartic acid, L-ornithine hydrochloride, and L-ornithine L-aspartate, with special reference to the ammonia level in serum.** Kyuji Abe, Yoshio Kowa, Schoichi Harigaya, and Takashi Morita (*Tokyo Research Laboratory, Tanabe Seiyaku Co. Ltd., Saitamaken, Japan*).

L-Aspartic acid and L-ornithine are members of the urea cycle. Several reports have appeared concerning the ammonia-detoxicating effect of these amino acids.

The present report is concerned with a comparative study of the activities of aspartic acid, ornithine, and other related compounds in suppressing the increase of blood ammonia levels caused by intraperitoneal injection of ammonium acetate into rats.

Since L-ornithine hydrochloride and L-ornithine, L-aspartate were the most effective of the amino acids tested, D-isomers of these compounds were also tested.

A comparison was also made between the activities of DL-isomers of aspartic acid, which are currently used for clinical purposes.

When given intraperitoneally, the D-isomers of ornithine hydrochloride and ornithine L-aspartate had little activity, whereas the corresponding L-isomers were effective. The D and L-isomers of aspartic acid were equally effective.

When given orally, the L-isomers of ornithine hydrochloride, ornithine L-aspartate, and aspartic acid were effective, while the D-isomers were not.

In these experiments the decrease of blood ammonia was accompanied by a simultaneous increase of urea concentrations in the blood. The most active compound in stimulating the urea formation was L-ornithine, L-aspartate followed by L-ornithine hydrochloride, indicating that the former is the most effective stimulator of the urea cycle.

**70. The influence of age on the amino acid level in blood plasma.** Richard F. Wehr, Valentine Moore, and George T. Lewis (*Department of Biochemistry, University of Miami School of Medicine, Miami, Fla.*).

There is doubt as to whether the requirements for and utilization of proteins and amino acids change with advancing age. In order to provide basic information for experiments designed to aid in a decision on this question, we have determined 19 amino acids in the blood plasma of young adults and of subjects over 60 years of age. Thirty-five individuals have been involved and in many instances repeat analyses have been carried out using a Beckman-Spinco automatic amino acid analyzer. Samples for analysis are now ultrafiltered after a 5-fold dilution with distilled water at a pressure of 375 psi at room temperature and in an atmosphere of nitrogen. The dilution results in somewhat elevated amino acid levels, due to less hold-up on the filters or perhaps pressure hydrolysis of peptides. Each sample is also analyzed for BUN and total α-amino nitrogen.

Results compare favorably with those obtained by other investigators except in the case of serine. For this amino acid values were found upwards of 300 μM/L. of plasma, which is of the order of twice previously recorded values. The possibility that glutamine may contribute to this result is under investigation.

Differences have been noted between young adults and aged individuals in plasma levels of threonine, half cystine, tyrosine, taurine, aspartic acid, and possibly glutamic acid.

No attempt has as yet been made to control the diet beyond the fact that the aged subjects are inmates of a local home for the aged and consume food prepared in a common kitchen.

**71. A routine procedure for screening of phenylketonuria in the newborn.** Paul Wong, Tohru Inouye, and David Yi-Yung Hsia (*Department of Pediatrics, Northwestern University Medical School, and Children's Memorial Hospital, Chicago, Ill.*).

Phenylketonuria is a hereditary metabolic disease caused by a deficiency of the enzyme, phenylalanine hydroxylase. The mental retardation of this condition can be effectively prevented if treatment with a diet low in phenylalanine content is started very early in infancy. The present paper will describe our experiences with a routine procedure for the screening of phenylketonuria used in 5000 newborn infants.

Described in detail are the modifications required to adopt the fluorimetric method of McCaman and Robins (*J. Lab. Clin. Med. 59*:885, 1962) for routine screening purposes using 0.1 ml. of capillary blood. The data will be analyzed in terms of sex, age, race, parity, maternal age, and birthweight and compared with observations on known phenylketonuric infants followed in a clinic and four other centers.

Since one laboratory technician can perform up to 100 determinations of serum phenylalanine daily, this approach using a quantitative method should serve as the best means of screening for phenylketonuria during the newborn period.

**72. Glutathione in anemias.** Niko Jesenovec and Marijana Fišer-Herman (*Institute for Clinical Chemistry, Zagreb, Domagojeva 2.I., Yugoslavia*).

An investigation was carried out to determine the concentration of oxidized and reduced glutathione of erythrocytes in anemias, using the glyoxalase method of Schroeder and Woodward and electrolytic reduction for GSSG.

In sideropenic, hemolytic, and some other types of anemias, average total glutathione and GSH values were reduced in comparison to normals, while GSSG concentrations were abnormally high. In cases of untreated pernicious anemia, GSSG averages were about 5 times those of normal erythrocytes, thus

increasing total glutathione concentrations to significantly higher values, in spite of somewhat lowered GSH.

A characteristic difference was found in the movements of glutathione in anemia, as compared to that in normal erythrocytes. Values in normals remained practically constant for months, but in the cells of anemia patients (and in some other diseased persons) very large fluctuations occurred within a few days. When GSSG was high, a fall of total glutathione to very low values usually followed promptly.

**73. Chromatographic studies of amino acids in sera and urine of patients with xeroderma pigmentosum and their normal relatives.** H. El-Hefnawi and M. F. S. El-Hawary (*Faculty of Medicine, Cairo University, and National Research Center, Nutrition Unit, Dokki, Cairo, U.A.R.*).

Chromatographic studies on amino acids in serum and urine of 24 xeroderma pigmentosum (x.d.p.) patients belonging to 18 families and their 83 apparently normal parents and family members, together with 15 other normal subjects with ages ranging from 4-50 years, were carried out. No pregnant females were included. Regulation and control of diet was adopted and rigidly supervised.

Two outstanding features in the urine of subjects with x.d.p. could be observed: a complex amino aciduria and the presence of an abnormal fluorescent spot which showed itself constantly in the area of tryptophan and valine spots. The amino acids present in the urine revealed a pattern which seems to be characteristic of the disease. The amino acids present in largest amounts are cysteine and cystine, histidine, lysine, arginine, aspartic acid, glutamine, serine, and alanine. Also a moderate increase above normal levels of glycine, glutamic acid, threonine, and hydroxyproline was found. Proline, tyrosine, phenyl alanine, and isoleucine are present in normal amounts. Valine and tryptophan have not been detected. It was noticed that the amino aciduria was equally manifested in the early as well as in the advanced cases of both children and adults.

A moderate amino aciduria, including more or less the same amino acids without the appearance of the abnormal fluorescent, occurred in about 25% of the apparently normal parents and other family members. In sera of x.d.p. patients and their relatives some amino acids spots were fainter in color than the corresponding ones in the control, while the other amino acids appeared normal in amount. However, an interesting phenomenon was the persistent appearance of the tyrosine spot in all the sera chromatograms of x.d.p. subjects. Tryptophan and methionine were encountered in sera chromatograms of only 12% of the x.d.p. subjects. Renal clearance studies have demonstrated that the amino aciduria in x.d.p. is tubular in origin.

The value of our findings is that chromatographic examination of urine and serum for their amino acid content can help in the differentiation between x.d.p.

and other clinically similar conditions which show photo-sensitivity and skin changes, particularly pigmentation of the exposed parts of the body.

## *SECTION B: ENZYMES I.*

**74. Some clinical experiences with isoenzymes.** A. L. Latner and A. W. Skillen (*Department of Clinical Chemistry, The Medical School and Royal Victoria Infirmary, Newcastle-upon-Tyne, England*).

Apart from the application of lactate dehydrogenase isoenzymes to diagnostic and other problems concerning myocardial infarction, isoenzyme studies have given results of value in other fields. With lactate dehydrogenase, appropriate abnormalities can be detected in the pre-icteric phase of infective hepatitis, and abnormal isoenzymes can occasionally be seen in the serum of patients suffering from cancer. Studies with leucine aminopeptidase isoenzymes are giving promising results in relation to the diagnosis of acute pancreatitis. Simultaneous determination of different groups of isoenzymes in the serum has provided parameters promising greater precision in technics. Studies with alkaline phosphatase have indicated that different isoenzymes exist in intestinal mucosa, liver, bone, kidney, and placenta. The major component normally present in human serum is apparently derived from liver and the placental component appears in the serum at the later stages of pregnancy. Alkaline phosphatase isoenzyme patterns in the serum seem to have some use in relation to differential diagnosis of jaundice. Starch-gel electrophoresis has also shown changes related to choline esterases in patients suffering from scholine apnea.

**75. Electrophoretic and other technics in the study of serum isoenzymes after myocardial infarction.** A. L. Latner and A. W. Skillen (*Department of Clinical Chemistry, The Medical School and Royal Victoria Infirmary, Newcastle-upon-Tyne, England*).

A brief account is given of visual technics after electrophoresis on starch-gel, as well as newer physical methods developed for detecting the appropriate changes in the serum without the use of electrophoresis. These latter have given results very comparable to the electrophoresis procedures, are simple to perform, and lend themselves to the multiple daily determinations required in the routine clinical chemistry laboratory. The newer technics involve the differential action of heat and the activity of organic precipitants. An interesting finding in serial observations after cardiac infarction has been the appearance of a "liver band" which cannot be explained by venous congestion and may be due to anticoagulant therapy.

**76. Experimental and clinical studies on β-glucuronidase isozymes.** Y. Yamamura, T. Aoki, T. Okochi, Y. Takahashi, and F. Ito (*The Third Department of Internal Medicine, University of Osaka School of Medicine, Osaka, Japan*).

Recently, enzymatic diagnosis with β-glucuronidases has been developed in the field of internal medicine. This report presents the distribution of β-glucuronidase in several organs and tissues in man, rats, and mice, and the patterns of β-glucuronidase in body fluids in patients with various diseases.

As enzyme preparation, crude extracts from several organs of man, rats, and mice were used. A purified enzyme preparation prepared by Fishman's method was also used. Separation of the isozymes from these materials was performed by using starch-block electrophoresis, or column chromatography with DEAE-cellulose.

Enzyme activity of each eluate was determined by using p-nitrophenyl-β-D-glucuronide as a substrate (Tsukamoto's method). Differences of each isozyme were investigated physicochemically, kinetically, and immunochemically.

Human, rat, and mouse livers were found to have β-glucuronidase which could be separated electrophoretically into 2–4 isozymes. β-glucuronidase in rat liver was clearly separated into three isozymes by column chromatography. In human sera from patients suffering from acute hepatitis, liver cirrhosis, and malignant tumor, high activity of β-glucuronidase was demonstrated electrophoretically in the position of β-globulin and albumin.

**77. Plasma N-acetyl-β-glucosaminidase and β-glucuronidase in health and disease.** J. W. Woollen and P. Turner (*Biochemistry Department, Royal National Orthopaedic Hospital, Stanmore, Middlesex, and Edgware General Hospital, Edgware, Middlesex, England*).

Fluorimetric methods, using 4-methylumbelliferyl substrates, were applied to the estimation of N-acetyl-β-glucosaminidase and β-glucuronidase in platelet-free plasma from a variety of subjects.

In normal subjects, the activities of both enzymes increased with age. β-Glucuronidase was higher in male than in female subjects. There was no significant variation in activity of either enzyme throughout the menstrual cycle.

In late pregnancy, increases in both activities, previously reported by other workers, were confirmed. In diabetics and in subjects with myocardial infarction within the previous 6 months, significant increases in N-acetyl-β-glucosaminidase were found in both sexes, but β-glucuronidase was raised only in females. Both activities were raised in all cases where myocardial infarction was of longer standing. One or both activities were increased in some types of liver

disease and malignancy, but were normal in other conditions, including most kidney and collagen diseases.

Considered together, the results suggest that the activity changes observed in diabetes and myocardial infarction are an earlier and more marked manifestation of those associated with ageing, while the findings in other conditions may reflect abnormal release of activity from a particular organ.

**78. Studies on the alkaline phosphatase of human urine.** D. W. Moss, P. J. Butterworth, and E. Pitkanen (*Department of Chemical Pathology, Postgraduate Medical School, London, W.12., England*).

Although human urine contains a considerable amount of acid phosphatase, the concentration of alkaline phosphatase is normally very low. The level of alkaline phosphatase may be increased, however, in a variety of pathologic conditions.

The occurrence of a raised urinary alkaline phosphatase has been studied, and starch-gel electrophoresis has been applied to the determination of the origin of the enzyme increment. Three distinct electrophoretic patterns have been observed. In the first, the urinary phosphatase appears to consist of filtered serum phosphatase, and in the second, the urine bands resemble the two fastest migrating alkaline phosphatase bands of kidney extracts; the third pattern shows an alkaline phosphatase zone migrating faster than any in the tissue extracts so far examined.

The significance of these alkaline phosphatase components in urine is discussed.

**79. Urinary excretion of alkaline phosphatase in normal and cirrhotic persons.** Albert A. Dietz (*Veterans Administration Hospital, Hines, Ill.*).

The urine and serum levels and the renal clearance rates of alkaline phosphatase were studied in normal subjects and in those with cirrhosis. In normal subjects, the mean serum level of alkaline phosphatase was 1.86 mM (SD, $\pm 0.54$) hydrolysis of *p*-nitrophenyl phosphate per liter per hour. In normals, the clearance rate of alkaline phosphatase was inversely related to the serum level, which could be expressed by the orthogonal regression equation $X_2 = 8.89 - 3.76X_1$, where $X_2$ is the clearance in milliliters per hour, and $X_1$ the serum level in mmoles per liter per hour. There was a minimal linear increase in clearance of alkaline phosphatase with increasing creatinine clearances in normal subjects. The renal clearance of alkaline phosphatase was not altered by water diuresis in normal or cirrhotic persons. In osmotic diuresis experiments using mannitol intravenously in edematous patients, there was a linear increase in the excretion rate of alkaline phosphatase with increasing urinary flows rates. This suggests that alkaline phosphatase is filtered and actively resorbed by the renal tubules. In patients with hepatic cirrhosis, the serum levels of alkaline phosphatase were significantly elevated. The mean value was 2.85 (SD, $\pm 1.83$) in patients with-

out ascites and 3.16 (SD, ±1.44) in patients with ascites. Both serum levels and clearance rates of alkaline phosphatase were normal in some patients with cirrhosis. Others had high clearance rates with normal serum levels, or normal clearance rates with high serum levels. The possible role of urinary excretion of alkaline phosphatase in following liver disease is discussed.

**80. La tréhalase du sérum sanguin.** Jean Emile Courtois, Marie Madeleine Debris, and Jean Claude Georget (*Laboratoire de Chimie Clinique de la Pharmacie de l'Hôpital Laënnec, Paris*).

Nous avons pu déceler dans les 400 sérums humains examinés la présence régulière de tréhalase. Cet enzyme scinde le tréhalose (α Glupyr-1→←1′ α glupyranoside) en 2 mol. de glucose. La tréhalase agit de pH 3.5 à 8.0 avec un optimum vers pH 5.3–5.5, elle se localise au niveau des $\alpha_2$ globulines, est assez stable par conservation à 4° et relativement thermostable. L'enzyme n'est pas inhibé par le glucose. Les hématies ont une activité infime et ne renferment pas d'inhibiteur de la tréhalase sérique.

Sérum (1 ml.) additionné de fluorure de sodium est mis en contact 6 hr. à 37° avec 1 ml. de tréhalose M/20 tamponné à pH 5.2. Le glucose libéré est déterminé par le réactif cupro-alcalin selon Nelson-Somogyi. Une unité d'enzyme (U.) correspond à une micromol. de tréhalose hydrolysé par minute pour 1 L. de sérum.

Les sujets normaux renferment 5 à 40 U., les valeurs les plus fréquentes sont de 10 à 15 U. Chez le même sujet l'activité varie légèrement d'un jour à l'autre; au cours de la journée elle augmente faiblement après les repas.

L'activité est normale dans la plupart des diabétiques avec assez rarement une nette augmentation. L'hyperglycémie provoquée par ingestion de glucose accroît faiblement la tréhalase.

Nous n'avons pas décelé de variations nettes dans diverses maladies virales, et dans les affections s'accompagnant d'une augmentation des transaminases dans le sérum.

Au cours des atteintes rénales on observe presque toujours des taux réduits (5 à 11 U.). Dans tous les cas de cirrhose et diverses affections hépatiques il y a diminution (2 à 5 U.).

Nous avons décelé de la tréhalase dans le sérum, l'intestin et le rein du Cobaye et du Lapin, mais l'activité du foie est infime. Chez le Rat nous avons observé comme Dahlqvist et Brun que le rat contient de la tréhalase dans l'intestin mais n'en renferme pas dans le rein.

Chez l'Homme le liquide céphalo-rachidien ne présente qu'une activité très minime tandis que la tréhalase du liquide d'ascite est de peu inférieure à celle du sérum.

L'urine a une activité très minime : 0.2 à 0.5 U.

Il existe de nettes différences entre les divers sujets et pour le même sujet au

cours de la journée (0.07 à 1.3 U.). Au cours des néphrites et néphroses l'activité tréhalasique urinaire paraît être supérieure à celle des sujets normaux.

**81. The clinical significance of synovial fluid acid phosphatase levels.** Jacob Kream, Martin A. Lehman, Catherine Kambolis, and Donato Brogna (*The Departments of Biochemistry and Pathology of the Laboratory Division, and the Department of Orthopaedic Surgery, Hospital for Joint Diseases, New York 29, N. Y.*).

Previous studies from this laboratory had demonstrated the presence of acid beta glycerophosphatase (ABGP) activity in synovial fluids obtained from patients who presented joint effusions. These studies suggested that ABGP activity was higher in the synovial fluids of patients with rheumatoid arthritis.

This report, which will present data substantiating previous findings, is based upon the determination of ABGP in synovial fluids and sera of 89 patients.

Synovial fluid ABGP has been found to be significantly elevated in those patients with rheumatoid and other inflammatory arthritides as compared to non-inflammatory arthritides. Serum levels were normal in all cases. No significant differences in the synovial fluid alkaline beta glycerophosphatase levels were observed.

Data will also be presented suggesting that ABGP originates from lymphocytes and the lining cells of the synovial membrane. These cellular elements are found in large numbers in the synovial fluids demonstrating higher enzyme activity. The findings are discussed.

**82. Lactic dehydrogenase isoenzymes in normal and disease states.** J. C. Nixon and G. F. Fitzgibbon (*National Defense Medical Center, Ottawa, Canada*).

The lactic dehydrogenase (LDH) isoenzymes of serum were fractionated by starch-gel electrophoresis and assayed on the gel using the nitro blue tetrazolium-coupled reaction. The intensity of color in each fraction was measured using a reflectance densitometer. Within certain limits the degree of color was proportional to enzyme concentration.

The LDH isoenzyme patterns were determined in the serum of 56 normal people. The units of LDH activity in each of the isoenzymes of the normals were as follows: $LD_1$, $22 \pm 14$; $LD_2$, $24 \pm 12$; $LD_3$, $52 \pm 17$; $LD_4$, $120 \pm 31$; and $LD_5$, $71 \pm 22$. In addition, single and multiple serum LDH isoenzyme fractionations were performed in a number of diseases including pericarditis, acute myelogenous leukemia, portal cirrhosis, infectious hepatitis, toxic hepatitis, nonicteric hepatitis, megalobalastic anemia, and myocardial infarction. The diagnostic significance of the LDH isoenzyme levels in these conditions is discussed.

**83. On the proteinases of malignant tumors.** V. N. Orekhovich and O. V. Kounina (*Institute of Biological and Medical Chemistry, Academy of Medical Sciences of the USSR, Moscow, USSR*).

Two cathepsins (termed cathepsins I and II) exhibiting maximal activity in acid media (*p*H 3.8) have been isolated from rat transplantable sarcoma M-I tissues.

Molecular weight, isoelectric point, as well as some other physical-chemical properties of cathepsins isolated were studied.

Terminal amino acids of both cathepsins were identified by means of a "finger-point" technic. Difference in primary structure of the enzyme molecules was established.

Difference in substrate specificity patterns of these cathepsins was revealed in experiments with protein substrates.

**84. L D H isoenzymes.** R. J. Wieme (*Interne Kliniek Akademisch Ziekenhuis, De Pintelaan, Ghent, Belgium*).

## *SECTION C: CALCIUM, MAGNESIUM, AND TRACE METALS*

**85. Determination of calcium-ion concentrations in the serum or plasma for clinical routine work.** Levi M. Mario and Elazar M. Silvio (*Biochemical Laboratory of the Institute of Hygiene and Social Medicine, Medical School, University of Sarajevo, Sarajevo, Yugoslavia*).

In the second decade of this century it was first demonstrated by means of dialysis that the plasma-calcium consisted of a diffusible fraction and a nondiffusible protein-bound fraction. The former consisted of an ionized fraction and a smaller complexed fraction (combined calcium with citrate, phosphate, etc.).

In recent years it was generally accepted that the ionized $Ca^{++}$ is physiologically the most active component. For this reason some investigators tried to find the most convenient method for estimating calcium ions. The first proposed method was a biological one, by McLean and Hastings in 1934. Rose developed a chemical method. Both are rather complicated for routine work in laboratories. The relationship on which the mentioned methods are based may be expressed as:

$$\frac{(Ca^{++}) \times (Prot.^{--})}{CaProt.} = K$$

However, Ludewig *et al.* found this equation unreliable and Sartori could not apply it to hypocalcemia. Bland offered another equation:

$$\text{Ca ions} = \text{Total calcium} - (0.87 \times \text{total protein})$$

This equation can be used only for normal conditions. In tetany or in hypopara-

thyroidism the calculated values for calcium ions are much lower than they actually are. The values obtained with McLean and Hastings's chart are above the actual values.

An equation and a nomogram covering the normal as well as the abnormal conditions have been developed. The equation is as follows:

$$Ca^{++} = \frac{254.11 - (62.89 \times \text{total protein}) + (60.16 \times \text{total Ca})}{100}$$

The nomogram is shown.

**86. A simple photometric instrument for the microdetermination of serum calcium.** Kurt M. Dubowski (*Department of Biochemistry, University of Oklahoma School of Medicine, Oklahoma City 4, Okla.*).

The direct complexometric microtitration of serum calcium, using metal indicators and conditions specific or highly selective for calcium, is potentially the procedure of choice. However, visual end point observation has not proved wholly satisfactory with most calcium-specific indicators, and the several instrumental approaches to such titrations have generally sacrificed simplicity of operation, or required expensive and complicated apparatus or graphic calculation of results. It therefore appeared desirable to develop a simple instrument system to permit the accurate and reproducible recognition of the end point in the direct complexometric titration of calcium ions with disodium ethylenediaminetetraacetate (EDTA) in the presence of several of the common indicators dyes. A practical instrument for this microdetermination has been developed, based upon the macrotitration system of Campbell (*Canad. J. Biochem. Physiol. 35*:1033, 1957). The unit is basically a specialized dual photocell filter photometer, with a microammeter as the end point indicator and associated controlled magnetic stirring and titrant delivery devices. This instrument has been successfully applied to the complexometric titration of micro quantities of calcium in blood serum by a slightly modified method of Bachra *et al.* (*Clin. Chem. 4*:107, 1958) using Cal-red as the indicator, with resultant enhanced speed, accuracy, and reproducibility.

Design and performance of the instrument are described, and results obtained by the instrumentally assisted microtitration procedure are compared with those yielded by conventional reference methods.

**87. A new EDTA method for the determination of calcium in blood serum.** William Richard Douglas (*Finseninstitutet Og Radiumstationen, Postboks 67, Copenhagen K, Denmark*).

Employing a new complexometric metal indicator for calcium, 2-(2′-thiazolylazo)-p-cresol, in combination with EDTA and cupric ions, it was possible to titrate directly the serum calcium in a strongly alkaline medium. The procedure

is based on calcium in the serum displacing copper from its EDTA complex as shown by sharp, distinct color change, unaffected by the presence of magnesium ions. Buffering was accomplished with potassium hydroxide, 1.25 M. The method is rapid, simple, and suitable for automation. No precipitation of proteins and no transfer of solutions are necessary. Interference by copper and iron may be obviated by addition of KCN. This new indicator was prepared by Bror Skytte Jensen of the Danish Atomic Energy Commission by coupling of diazotized 2-aminothiazole with p-cresol at 0°. Recoveries were 97–101%. Results are compared with the standard method of Kramer and Tisdall as modified by Clark and Collip.

**88. Étude de la citraturie, particulierement dans la lithiase renale.** P. Desgrez, J. Thomas, E. Thomas, and H. Rabussier (*Laboratoire de Chimie, Faculté de Médecine, Paris, 6e, France*).

Le rôle joué par la concentration des citrates dans les urines a été fréquemment mis en cause au cours des recherches portant sur les facteurs prédisposant à la formation des calculs.

Nous avons pu confirmer les variations importantes de l'élimination de l'acide citrique chez le sujet normal. La dispersion est telle que les chiffres extrêmes se situent entre environ 200 et 1000 mg./24 hr. avec une valeur moyenne à 715 mg. Nous avons noté après d'autres auteurs une nette diminution de la citraturie par un traitement acidifiant, une augmentation aussi importante par un traitement alcalinisant.

Plus de 400 dosages affectués sur des sujets atteints des différentes variétés chimiques de lithiase rénale, nous ont permis les constatations suivantes: en cas de lithiase oxalique, surtout au début de l'affection et en l'absence d'infection, la citraturie est normale. Lorsque la lithiase est ancienne la citraturie est par contre fréquemment abaissée, la moyenne tombant à 277 mg./24 hr. En cas de lithiase urique le comportement de la citraturie est sensiblement le même qu'en cas de lithiase oxalique. Par contre, dans la lithiase phosphatique, l'élimination d'acide citrique est le plus souvent basse et même pratiquement nulle dansles formes avancées; la moyenne se situe à 53 mg./24 hr. avec des extrêmes variant de 0–241 mg.

Une étude systématique des différents cas observés nous a montré que deux facteurs interviennent pour faire baisser le taux des citrates urinaires:

1. L'infection est l'élément le plus fréquemment responsable et les dosages successifs sur urines infectées montrent la disparition rapide et totale des citrates: l'adjonction d'antiseptiques mercuriels ralentit la destruction des citrates, mais ne l'arrête pas totalement.

2. L'insuffisance rénale est le deuxième élément responsable des hypocitraturies: son influence apparait surtout dans les cas de lithiases avec insuffisance rénale et sans infection; les dosages montrent des taux bas qui se maintiennent

pendant plusieurs jours : preuve indirecte d'une élimination faible dès le stade tubulaire.

La destruction des citrates d'origine infectieuse conduit à l'acide carbonique et aux carbonates neutres ou acides, ainsi que nous l'avons vérifié par le dosage, à l'appareil de Van Slyke, du $CO_2$ à partir de solutions citratées, ensemencées avec des bactéries isolées d'urines infectées. Cette disparition du citrate, élément important du tampon acide des urines explique leur alcalinisation plus facile et ajoute ses effets à l'ammoniogénèse uréolytique.

Aussi la baisse des citrates urinaires ne peut être évoquée comme facteur essentiel de la lithogénèse puisqu'elle est incontestable et n'existe pas au début de la maladie, au moins dans la variété oxalique des lithiases calciques. Tout au plus peut-elle représenter un facteur aggravant secondaire.

**89. The role of citrate in the metabolism of divalent cations.** Samir Hanna (*Clinical Pathology Department, Faculty of Medicine, Ein Shams University, Abbassiah, Cairo, U.A.R.*).

Parathyroid hormone and growth hormone produce effects which resemble the effects of administration of vitamin D. The finding of a rise in plasma citrate level in acromegalic subjects as well as after the administration of parathyroid hormone or growth hormone emphasizes the similarity between the effects of growth hormone, parathyroid hormone and vitamin D.

The effect of vitamin D on gut, plasma, and urinary citrate was studied, and it was found that changes in citrate may be the responsible reason for changes produced in Ca and Mg metabolism. Studies on glucocorticoids will be presented to show that they exert effects on both citrate and divalent cations that are diametrically opposite to those of vitamin D.

**90. Plasma magnesium levels in children.** Samir Hanna (*Clinical Pathology Department, Faculty of Medicine, Ein Shams University, Abbassiah, Cairo, U.A.R.*).

Studies of plasma magnesium levels have not yet been widely introduced to the field of routine clinical chemistry. However, the old methods of estimating magnesium are now superseded by simple flame-photometric technics.

The author has studied normal plasma magnesium levels in adults and in various cases of hypomagnesemia. In this paper a survey of plasma magnesium levels in normal children and in various pathologic conditions (rickets, renal tubular defects, diarrhea) is presented.

**91. Normal occurrence of selenium in food and body tissues.** H. H. Tausky and A. T. Milhorat (*Institute for Muscle Disease, New York 21, N. Y.*).

Until relatively recently, interest in selenium as a biologically active material was confined to its toxicity in accidental poisoning in humans, and to a stock

disease caused by ingestion of forage and cereals grown in soils rich in this element. During the past decade evidence has accumulated that selenium may be essential for certain metabolic processes. However, information on the selenium content of foods, its distribution in the tissues of the body, and the output in the urine has been lacking, no doubt in part because no suitable method for the determination of selenium was available. The studies reported on show that the average normal diet contains appreciable amounts of selenium, and that most of it is bound to protein. Eggs contain significant amounts of selenium, the highest concentration being in the yolk. The selenium content of cereals can be high depending on the amounts of selenium contained in the soil.

Selenium was found in all tisues of the mouse, rabbit, and chicken with apparent accumulation in kidney, liver, and hair. Most of the selenium in the blood of the mouse and rabbit was found in the red cells. Humans excrete significant amounts of selenium in urine and these appear to be related to the selenium content of the diet. Small amounts of selenium injected into eggs and chickens produced no toxic effect; however, gradually increasing doses established a rather critical level above which toxicity always was observed, and below which the material produced no ill effects.

Our method for determination of selenium in biological fluids and tissues is described.

**92. Determination of microgram quantities of copper in biological material.** Ramon Stoner and Waldemar Dasler (*Department of Biochemistry, The Chicago Medical School, Chicago, Ill.*).

A stable, sensitive method for the determination of copper in biological material is described whereby 0.5–2.0 $\mu$g. may be estimated with conventional laboratory equipment.

The organic matter is first destroyed with a nitric-perchloric acid mixture. The inorganic residue is neutralized with sodium hydroxide.

Sodium citrate and sodium carbonate are added and the copper is then extracted from this solution with a benzene solution of 1,5-diphenyl-carbohydrazide. A stable color is formed in the benzene layer with an absorption maximum at 540 m$\mu$ and a molar absorptivity of about 55,000. The benzene layer is removed and read in a Coleman spectrophotometer.

**93. An improved serum iron measurement with sulfonated bathophenanthroline.** Roger Callahan (*Research and Medical Services, Veterans Administration Hospital, and the Department of Medicine, University of Oklahoma School of Medicine, Oklahoma City, Okla.*

A simple modified procedure for serum iron has been developed. The protein-bound iron in 1.0 ml. of serum is dissociated with 1.5 ml. of 0.2N HCl in the presence of a strong reducing agent (in this case, 1 drop of thioglycholic acid),

as in the method of Peters. The protein is precipitated with 0.5 ml. of 30% trichloroacetic acid. 2.0 ml. of the resulting supernatant is then reacted with 1.0 ml. of 0.05% 4,7-diphenyll, 10-phenanthrolinedisulfonic acid disodium salt in 54% potassium acetate solution. The optical density is read at 535 mμ and is found to follow Beer's law over a concentration range of 50–600 μg./100 ml. The extraction method of Peters is used because it gave the highest and most consistent recoveries of serum iron.

4,7-Diphenyl-1, 10-bathophenanthrolinedisulfonic acid, disodium salt is the colorimetric reagent of choice because, in addition to being soluble, it is very specific and sensitive in its reaction with iron. Potassium acetate was used instead of saturated sodium acetate because its greater solubility allowed a one-solution colorimetric reagent. These reagents are quite stable over a period of several months. Recovery of added serum iron was from 97.5 to 102%. Duplicate samples of serum determined by this new procedure were compared to other standard methods. Comparable results were obtained in all cases, but the new procedure gave greater precision and, except for the method of Peters, better recoveries.

**94. Determination of iron in serum using solvent extraction.** D. T. Forman, H. A. Woodson, and S. Jocher (*Department of Pathology, Mercy Hospital, and Department of Biochemistry, Northwestern University Medical School, Chicago, Ill.*).

The spectrophotometric method for the determination of iron in serum with bathophenanthroline (4,7-diphenyl-1, 10 phenanthroline) was modified by extracting the iron complex into a chloroform-ethanol mixture. Thus, interferences from colored ions such as chromium and cobalt are eliminated by the liquid-liquid extraction procedure and errors in the absorbance measurement caused by the presence of a colloid or precipitate during the color development are avoided. The chloroform-ethanol extraction system was selected because the organic phase is heavier than the aqueous phase and can be readily separated. Dilution of the chloroform solvent with ethanol is advantageous because it clears any turbidity in the organic phase caused by small droplets of water. Varying the ethanol-chloroform ratio from 1 :1 to 1 :4 has little effect on the color system. Iron (up to 8 μg.) is completely extracted within about 30 sec. into 1.5 ml. of the chloroform-alcohol mixture. The colored complex is stable in the chloroform-ethanol solution for at least 1 week. The small volume of organic phase effectively concentrates the iron complex and therefore affords increased sensitivity. This method can be used for determining iron in 0.1–1.0 ml. of serum. The advantages of this method are its sensitivity and specificity. The effects of variations in the experimental conditions are examined. Results of studies on the accuracy and precision of this modified technic showed agreement with other methods.

## *SECTION D: METHODS AND NORMALS*

**95. Recent concepts in clinical microchemistry.** Samuel Meites (*Children's Hospital, Columbus 5, Ohio*).

In recent years, two concepts of clinical microchemistry have emerged: the micro-macro and the micro-micro approaches. These are distinguished on the basis of volumetric considerations in the pipetting of the specimen and in the pipetting of reagents. Which of the two is the most promising? Need both be used? Answers may be sought provided the approaches are given tentatively exact definitions. In the micro-macro system, the pipetting of the specimen alone is confined to the micro (less than 0.5 ml.) or ultramicro (0.05 ml. or less) range, and is carried out with a TC pipet (A.C.S. specifications), into as large a dilution as possible. Subsequent steps are performed with TD macropipets (N.B.S. specifications), with volumes of 0.5 ml. or greater. The micro-micro system is characterized by the use of TD micro- or ultramicro pipets for all steps. There are advocates of both approaches. Preliminary investigation leads to the conclusion that the micro-macro approach is the most practical of the two on the basis of greater pipet accuracy and greater economy of laboratory space and equipment. This problem and others pertaining to clinical microchemistry are discussed. Consideration will be given to the use of scaling-down, alternate chemical reactions, and newer physical methods of analysis in microchemistry.

**96. Determination of creatine and creatinine in urine.** Andre C. Kibrick and Ade T. Milhorat (*Institute for Muscle Disease, New York 21, N. Y.*).

It is shown that the increase in color in the Jaffe reaction produced by non-creatinine impurities in urine is of minor importance ordinarily, and that elimination of procedures for purification not only simplifies the determination, but actually leads to greater precision. Conversion of creatine to creatinine is performed in a household pressure cooker in the presence of picric acid. Although Beer's law is followed precisely over a wide range of concentration with an interference filter of 519 mμ, a straight-line relationship is also obtained with a Coleman spectrophotometer at 510 mμ. The precision that is possible by this simple method permits detection of minor changes in the creatine content of urine.

Data are presented to compare results obtained in many samples of urine under the following conditions:

1. Heating in a water bath and in a pressure cooker
2. Determination of color of the Jaffe reaction with and without extraction with ether
3. Estimation in the spectrophotometer and in a photoelectric colorimeter with the interference filter

Most of the data are on patients on creatine-free diets who excrete high and

low amounts of creatine. However, there are some experiments on normal subjects on regular diets to show that even under these conditions ether extraction can be eliminated.

**97. The urinary excretion of creatine and creatinine by children in hospital.** Sanford H. Jackson and Donald M. Mills (*Research Institute, The Hospital for Sick Children, Toronto, Ontario*).

Not infrequently the quantitation of urinary constituents is expressed in units relative to the creatinine excretion (i.e., milligrams per gram of creatinine). This stratagem is used to circumvent the difficulty of collecting reliable 24-hr. urine specimens, particularly in children. It presupposes a constancy of urinary creatinine excretion that has not been adequately verified. The institution of a metabolic ward and a urine collection ward in this hospital, both of which are equipped and expertly staffed to assure exact collections, has made it possible to determine urinary creatine and creatinine excretion in a hospital population.

The results show that, in a given individual, the daily excretion varies within $\pm 10\%$ of the mean, but that there may be occasional fluctuations of as much as $\pm 50\%$. The variations between individuals, when calculated either on a weight basis or a surface area basis, are so great as to negate the usefulness of either total creatinine (creatine plus creatinine) or preformed creatinine as a basis for quantitation of other urine constituents.

**98. Age variations of NPN and urea of newborn infants.** Bertil Josephson and Olle Järnmark (*Central Clinical Laboratory, St. Erik's Hospital, Stockholm, Sweden*).

At birth the NPN of the child is approximately identical with that of the mother, whereas serum urea is considerably lower. Within a few days the NPN has also decreased to subadult values. At an age of 4–6 months, the concentration figures increase to adult values. The explanation is discussed.

**99. Plasma PSP index of renal function (PSPI).** M. Henry Gault, T. Douglas Kinsella, and B. Koch (*Department of Clinical Chemistry, Queen Mary Veterans' Hospital, and Departments of Endocrinology and Metabolism and Urology, Royal Victoria Hospital, Montreal, Canada*).

To avoid the common problems of incomplete or inaccurately timed urine collections, a renal function test which eliminates the need for urine specimens has been developed.

Following intravenous injection of PSP (1 mg./kg.), serial blood specimens are taken from a heparinized plastic infusion set at 5-min. intervals between 15 and 35 min. After the phase of rapid dilution (0–10 min.), a period of exponen-

tial fall dependent on renal function is obtained for at least 20 min. The rate of fall in plasma concentration during this phase is used as an index of PSP clearance (PSPI).

Plasma PSP is measured by a simple, rapid, colorimetric method. Proteins are precipitated with acetone and the alkalinized supernatant is read at 560 mμ.

Data from 175 tests show no patient to have had an elevation in BUN or serum creatinine, or a significant lowering of creatinine clearance, unless the PSPI has been reduced. The correlation coefficient between the PSPI and the urine 15-min. PSP excretion is 0.96 in 70 normal patients, compared to low correlation in patients with voiding problems. The correlation coefficient with PAH clearance is 0.91 (16 patients), and with creatinine clearance, 0.87 (75 patients). In 25 patients the average difference between values found on two occasions was 7%. The index is independent of dose in the range 0.5–2 mg./kg., indicating it is a function of PSP clearance.

The PSPI provides a sensitive and reproducible renal function test based solely on plasma samples.

**100. Cholesterol and phospholipid in human cerebrospinal fluid.** Yung Soo Shin (*Department of Clinical Pathology, St. Mary Mercy Hospital, Gary, Ind.*).

Interest has been focused recently on the cholesterol and phospholipid levels in the CSF of individuals suffering from degenerative disorders of the CNS or other conditions producing an alteration of the blood-CSF barrier permeability.

This study is concerned with silicic acid chromatography determination of cholesterol, esterified cholesterol, and phospholipid in the CSF of 132 normal (non-neurological) individuals of varied age and sex. The values obtained were 0.472, 0.255, and 0.538 mg./100 ml., respectively, in males, and 0.453, 0.256, and 0.515 mg./100 ml., respectively, in females. Although no significant age and sex differences were noted, in younger individuals (1–10 years) total cholesterol was slightly lower, and cholesterol esters and phospholipids were higher; total cholesterol was higher for ages 51–60. The level of inorganic phosphorus was remarkably unchanged (by age and sex differences), and in inflammatory or degenerative diseases (CNS) was minimally or not at all elevated.

Ratios of cholesterol to phospholipids, cholesterol ester to total cholesterol, and lipid phosphorous to inorganic phosphorus were also estimated, average normal values being $0.88 \pm 0.29$, $0.55 \pm 0.08$, and $0.014 \pm 0.002$, respectively; observations suggest that cholesterol/phospholipid may increase, while cholesterol ester/total cholesterol declines as age advances.

Cholesterol and phospholipid were also determined in a few infectious, neoplastic, and functional CNS disorders, and a "possible" diagnostic value of lipid P and inorganic P ratio is suggested, although the number of cases is too small (12 cases) for any definite conclusions.

**101. Enzymatic determination of glycerol in the measurement of triglycerides.** F. H. Kreutz (*Medizinische Universitätsklinik, Marburg, Germany*)

A method has been developed for the enzymatic determination of glycerol by coupling the following reactions (*Klin. Wochschr. 40:*362, 1962):

$$\text{Glycerol} + \text{ATP} \xrightarrow{\text{glycerolkinase}} \text{L}(-)\text{glycerol-1-phosphate} + \text{ADP} \quad (1)$$

$$\text{ADP} + \text{phosphoenolpyruvate} \xrightarrow{\text{pyruvatekinase}} \text{ATP} + \text{pyruvate} \quad (2)$$

$$\text{Pyruvate} + \text{DPNH} + \text{H}^+ \xrightarrow[\text{dehydrogenase}]{\text{lactic}} \text{lactate} + \text{DPN}^+ \quad (3)$$

$$\text{Glycerol} + \text{phosphoenolpyruvate} + \text{DPNH} + \text{H}^+ \longrightarrow \text{L}(-)\text{glycerol-1-phosphate} + \text{lactate} + \text{DPN}^+ \quad (4)$$

In the over-all reaction one mole of DPNH is oxydized per one mole of glycerol phosphorylized. Under the conditions used, Reaction 4 proceeds virtually to completion and the change in optical density at 340 mμ is a direct measure of the amount of glycerol in the assay. Unlike other enzymatic glycerol methods (Wieland, *Biochem. Z. 329:*313, 1957, Hagen and Hagen, *Canad. J. Biochem. 40:*1129, 1962), the reaction is complete in less than 5 min. due to the favorable equilibrium of the LDH-catalized step. Glycerol concentrations as low as $2 \times 10^{-8}$ moles/ml. can be made either directly on 0.5 ml. of serum or on perchloric acid extracts.

Utilizing the enzymatic glycerol determination, measurement of total serum glycerol can be done directly on alkaline hydrolysates of 100–500 μl. serum without any preliminary extraction procedures. Serum triglyceride concentrations measured by this method were in good agreement with values calculated from chemical determinations of total esterified fatty acids, cholesterol esters, and phospholipids.

**102. Simultaneous determination of serum barbiturates and salicylates by ultraviolet spectrophotometry.** Solveig Bjerre and C. J. Porter, with the technical assistance of Ignatia Leewens (*Division of Chemistry and Biochemistry, Department of Laboratories, Toronto General Hospital, Toronto, Canada*).

The finding of both salicylates and barbiturates in serum is a frequent occurrence, and measurement of barbiturates in the presence of salicylates by U.V. spectrophotometry leads to serious errors.

The U.V. spectrophotometric method described employs a recording Beckman DB spectrophotometer and is based on the fact that, while barbiturates have no absorption at wavelength 296 mμ, salicylates have an absorption peak which is proportional to their concentration. Barbiturates are detected by their charac-

teristic absorption peak at 239 mμ (*p*H 10) and then determined from the difference in optical density at *p*H 10 (first ionization) and *p*H 1 (un-ionized). Salicylates have a high optical density at both *p*H values, and their interference with the detection and determination of barbiturates can be calculated and corrected for.

Both salicylates and barbiturates are quantitatively extracted into chloroform followed by re-extraction into borate buffer (*p*H 10). By this method, salicylates and barbiturates or a mixture of both, can be determined quantitatively in the same extract.

**103. Interference of thiouracil in the Zak cholesterol method, and elimination of this interference with silver iodate.** Eugene W. Rice (*Singer Research Laboratory, Allegheny General Hospital, Pittsburgh, Pa.*).

It is common practice to determine both butanol-extractable iodine and cholesterol on a given sample of serum. Recently Man proposed adding thiouracil in approximately 0.001 M concentration to stored sera as a preservative for butanol-extractable iodine (*J. Lab. Clin. Med. 59*:528, 1962). Investigation has revealed that the Zak ferric chloride-sulfuric acid color reaction for cholesterol gives erroneously low results in the presence of thiouracil. Addition of 0.13 mg. of thiouracil per milliliter of serum (approximately 0.001 M) causes an average decrease in the apparent cholesterol value of 30–40 mg./100 ml. by suppressing the 560 mμ absorption maximum of the color complex.

Further studies showed that this thiouracil-cholesterol color inhibition may be overcome by simply shaking the serum for about 5 min. with solid silver iodate (25–50 mg./ml.) prior to extraction with ethanol-acetone. Fortuitously, this is the identical treatment previously developed to eliminate the enhancement of the Zak cholesterol color reaction caused by the presence in sera of exogenous bromide or iodide (*Clin. Chem. 3*:160, 1957).

**104. Gas chromatographic determination of individual 17-ketosteroids in urine: Studies on normal human subjects.** E. H. Keutmann, Mario Sparagana, and W. B. Mason (*The University of Rochester Medical Center, Rochester, N. Y.*).

A procedure developed by the authors permits quantitative determination of etiocholanolone, androsterone, dehydroepiandrosterone, 11-ketoetiocholanolone, 11-hydroxyetiocholanolone, and 11-hydroxyandrosterone in urine with a rapidity, accuracy, and ease hitherto impossible. 4-Androsten-11β-ol-3, 17-dione is added as internal standard prior to hydrolysis, and an extract is prepared by conventional steps. Two portions of the extract, one from which dehydroepiandrosterone has been precipitated with digitonin, are analyzed by gas chromatography using a SE-30 column. The amounts of individual ketosteroids are cal-

culated relative to the internal standard. The method is described and data are reported for a series of normal people.

## *FRIDAY, AUG. 23*

### *SECTION A: ENZYMES II.*

**105. Studies on the enzyme secretion of the human pancreas.** W. Rick (*Medizinische Universitätsklinik, Giessen, Germany*).

The function of the exocrine pancreas in man can be measured by the following technic. Quantitative drainage of the duodenal juice is performed by use of a double balloon tube (Bartelheimer) which closes the duodenum towards the stomach and the jejunum. After a period of spontaneous secretion, secretin and pancreozymin are injected intravenously. Specific and sensitive methods are used for the determination of trypsin, chymotrypsin, carboxypeptidase A, amylase, and lipase. The enzyme concentrations, in international units per milliliter, are converted to enzyme secretion rates in international units per minute. As crystalline enzymes from human pancreas are not available, crystalline beef trypsin, chymotrypsin, carboxypeptidase A, and pig pancreas amylase can be used as standards, with the differences in the specific activities of enzymes from different species kept in mind.

In normal persons, the data for the spontaneous secretion showed a high degree of statistical scatter. After injection of secretin less variability was seen. The narrowest range of normals was found following stimulation with pancreozymin; therefore these values were taken as criteria for pancreatic secretory function. In health the different enzymes are secreted in constant proportions (Babkin, Lagerlöf). In contrast to these results on normal subjects a selective reduction of certain enzymes in chronic pancreatitis was often found. In some cases, lipase exclusively was diminished, while in other patients lipase, trypsin, and chymotrypsin were secreted at a subnormal rate. The findings were compared with observations made at operation and by histologic examination of biopsy specimens, and the diagnostic value of the procedure outlined above was indicated.

**106. Lipase-tributyrinase of breast milk and gastric digestion of newborns.** B. M. Soumtsov (*Biochemical Laboratory, SRIOG, Moscow G-2, U.S.S.R.*).

The lipase of breast milk is an extracellular enzyme of digestion which serves not growth needs but the improvement of gastric lipolysis of the child. To comprehend the physiology and biochemistry of digestion of the milk fat of newborns it is important to know how lipolytic activity of milk changes because of physiolog-

ical shift connected with the dynamics of lactation, age, etc., and also with the seasons. The tributyrinase of breast milk was investigated; in accordance with Freudenberg, it is identical with lipase but differs from the latter by not requiring the preliminary substrate activation. A phenomenon of inadequate decrease of tributyrinase activity in multiple dilutions of milk was discovered; this phenomenon is favorable probably to progressive lipolysis in the newborn stomach. Periods of lactation and the age of mothers, as well as the level of their lactation were not found to have any regular influence on enzymatic activity. However in the autumn-winter period the tributyrinase activity decreased to 80 $E_{18}$, but in the winter-spring period it increased to 105 $E_{18}$. On the other hand women treated with ascorbic acid also showed the statistical decrease of milk tributyrinase from 110 to 80 $E_{18}$. The lipolytic activity of breast milk not only depends on the level of vitamin C in nutrition, but itself may be regarded as an original "vitamin." This "vitamin" is of a great importance for early periods of postnatal ontogenesis of the human being; actually the lipase of breast milk is a biologically active substance which is not practically synthesized by the newborn, but is taken in with food. In contradistinction to the majority of vitamins the need for "vitamin-Lipase" is exhausted evidently at the age when the newborn begins to receive mixed feeding. Our thesis is not only of terminological but also of practical importance, because the further investigation of milk tributyrinase must coordinate with the principles of vitaminology. It is necessary to establish the daily requirements for this "vitamin" for the newborn and to raise a question about its addition to milk formulas as well as in enriched food, etc.

**107. Separation of amylase from human serum and urine by gel filtration and the demonstration of an abnormal binding of serum amylase by a γ-globulin.** P. Wilding, W. T. Cooke, and R. Gaddie (*The General Hospital, Birmingham, England*).

A technic for the study of amylase in serum, urine, saliva, and pancreatic juice is described. The various specimens were subjected to gel filtration on a column of dextran gel (Sephadex G.100) and the points of elution of amylase in the consecutive fractions of eluate determined. The fractions having amylase activity were further examined by electrophoresis and immunoelectrophoresis.

Serum from 6 healthy adults, four patients with acute pancreatitis, 3 patients who had undergone total pancreatectomy, and two patients with mumps was fractionated by gel filtration. Fractions of eluate were collected and analyzed for protein content and amylase activity. The results obtained showed that the amylase in all these sera was present as a separate moiety not bound to any serum protein. The experiment also demonstrated that amylase from all the abovementioned serum specimens and from urine and pancreatic juice are similar with respect to molecular weight and electrophoretic mobility.

Evidence is presented of a rare case in which there were persistent high levels

of serum amylase due to binding of serum amylase by a high molecular-weight γ-globulin, with little or no excretion of amylase in the urine.

**108. Electrophoretic distribution of amylase activity.** Matsamitsu Kawaguchi, J. Edward Berk, Ichiro Ujihira, Rashid Zeineh, and Ronald L. Searcy (*Departments of Medicine and Pathology; Los Angeles County General Hospital (Unit 2) and California College of Medicine, Los Angeles, Calif., and Department of Medicine, Sinai Hospital of Detroit and Wayne State University College of Medicine, Detroit, Mich.*).

Serum amylase activity appears attributable to several isozymes elaborated by liver, salivary glands, pancreas, and other tissues. To elucidate further the amylase enzyme complex, amyloclastic patterns of normal and pathologic serum in man, normal serum in rabbits and dogs, and pancreatic secretion and cyst fluid in man were determined electrophoretically.

Duplicate aliquots of material applied to filter paper were subjected to electrophoresis for approximately 16 hr. One strip was stained for protein with bromphenol blue. The other strip was placed on a starch-agar block for 24 hr. at 37°. After incubation, the paper was removed and the agar block flooded with dilute iodine solution. Colorless zones against a blue background indicated areas of starch hydrolysis. In normal human serum, amyloclastic activity was largely confined to a zone near the point of specimen application. Much smaller amounts of starch hydrolysis were observed in electrophoretic areas corresponding to albumin, whereas beta-globulin fractions contained only trace activity. Sera from patients with acute pancreatitis exhibited increased amyloclastic activity solely in the gamma-globulin zone. These findings were consistent with amyloclastic patterns obtained by electrophoresis of pancreatic secretion and pancreatic cyst fluid.

Normal serum, pancreatitis serum, and cystic fluid electropherograms were cut into segments. Amylase activities of filter paper extracts were measured spectrophotometrically in terms of maltose liberation. These data demonstrated a quantitative distribution of amylase activity similar to the qualitative starch-agar patterns. It is noteworthy, that no electrophoretic protein fraction was completely devoid of amylase activity.

**109. Cholinesterase activities.** Marjorie Bick (*Alfred Hospital, Victoria, Australia*).

Cholinesterase activity has been determined in plasma and erythrocytes collected from members of a small rural population of orchardists exposed to organophosphorus sprays. The substrates used were those of Frawley *et al.*, and the method that of Caraway adapted to the AutoAnalyzer by Winter. The mean value for the cholinesterase in plasma was 169 μM acetylcholine hydrolyzed per

milliliter of plasma per hour, that of the control group 166. The mean value for the acetyl cholinesterase in the erythrocytes from the orchardists was 277 $\mu$M/ml./hr., and from the control group, 320 $\mu$m/ml./hr.

The values obtained after one month's spraying fell to 150 $\mu$M/ml./hr. for plasma, and 218 $\mu$M/ml./hr. for erythrocytes. In a total of 85 persons from 63 families, in 16, the fall in erythrocyte cholinesterase was accompanied by a fall in plasma cholinesterase, but in 19 individuals there was no fall in plasma cholinesterase corresponding to the fall in acetylcholinesterase. A fall in cholinesterase but not acetylcholinesterase was observed in 4 persons. All plasma samples showed inhibition by dibucaine. Low values for cholinesterase and acetylcholinesterase were found in 2 samples.

Cholinesterase has been determined in plasma obtained from surgical patients, excluding any that were jaundiced, preoperatively and 24 hr. after anesthesia and the use of succinylcholine as a muscle relaxant. The mean value obtained was 129 $\mu$M/ml./hr. and no fall was observed in the preoperative level.

**110. Glucose-6-phosphate dehydrogenase deficiency on Curacao (Netherlands Antilles).** H. Schouten and A. van der Sar (*St. Elizabeth Hospital, Breedstraat 193(O), Willemstad, Curaçao, N.A.*).

On Curaçao, G6PD deficiency has been found among several groups of people including hospital and psychiatric patients, diabetics, lepers, blood donors, and people with abnormal hemoglobin. Besides a screening test (brilliant cresyl blue-reduction, Motulsky) quantitative technics were used for G6PD (Zinkham), the glutathion stability test (Stevenson), and the formation of Heinz-bodies (Beutler). Normal values are reported. The screening test appears to be of lesser value in cases of abnormal hemoglobin, because of a higher G6PD level in these subjects. The GSH stability test is the recommended one. Case reports are made of blood transfusion reactions, hematemesis, and hemolytic jaundice of the newborn because of G6PD deficiency. Family trees show that the defect is inherited as an incomplete, sex-linked, dominant gene with variable expression in the females. According to Kraus and Beutler, also intermediate forms in the males are likely to be present. The island of Curaçao is divided into three districts, one of which has a higher incidence of G6PD deficiency. This might support earlier theories about the origin of the natives.

**111. Isolation and properties of an esterase from hog liver microsomes.** Klaus Krisch (*Physiologisch-chemisches Institut der Universität, Giessen, Germany*).

In hog liver microsomes an enzyme is found which hydrolyzes the amide bond of acetanilide. The isolation of this enzyme—provisionally named "acetanilide-amidase"—in six steps is described. The purification as related to microsomes is about 75-fold, the yield being about 20%.

According to chromatographic, electrophoretic, and ultracentrifugation experiments the enzyme can be considered as an essentially homogeneous protein. The enzyme is inhibited by a number of typical esterase inhibitors. The investigation of the substrate specificity shows that, besides acetanilide, certain other amides (L-leucyl-β-naphthylamide, monoethylglycine-xylidide) are also split. The enzyme, however, does not attack proteins, dipeptides, and L-leucinamide. Aliphatic esters and procaine (but not acetylcholine) are readily hydrolyzed by the isolated enzyme. The turnover number for tributyrin and ethylbutyrate is about 20,000 (related to 100,000 g protein, t = 37°). In contrast, acetanilide is hydrolyzed at a considerably slower rate (turnover number about 50). The isolated enzyme is very probably identical with the long-known ali-esterase (unspecific esterase, B-esterase) of liver representing the first homogenous preparation of this enzyme.

The pure esterase hydrolyzes L-leucyl-β-naphthylamide. Therefore this compound is not a specific substrate for the determination of the so-called leucine aminopeptidase activity in serum or in histochemical experiments.

***BILIRUBIN***

**112. Different effects of light on the bilirubin of jaundiced serum.** S. H. Blondheim and N. Kaufman (*Metabolic Unit and Laboratory, Hadassah-University Hospital, Jerusalem, Israel*).

Light produces at least four changes in jaundiced serum and bilirubin-albumin mixtures. The total bilirubin concentration decreases as bilirubin is destroyed and biliverdin formed. There is a spectral change, with shift in the maximal absorption from 450–460 mμ to 420 or even lower. There is a 2- to 3-fold increase in the direct diazo reacting fraction, and a yellow pigment is produced which is ultrafilterable, has an absorption peak at 420 mμ, and gives a very weak diazo reaction.

Under conditions to be described, irradiation produced direct-reacting bilirubin from unconjugated bilirubin in albumin soluton without either a decrease in bilirubin concentration or shift in the absorption spectrum.

In serial spectrophotometric studies of the sera of patients recovering from infectious hepatitis, the absorption maximum shifted progressively from 450 to 420 mμ as the total bilirubin decreased. The pigment(s) absorbing at 420 mμ, which are present in normal serum as well, are apparently not heme pigments. This was shown by correction of the spectral curves for the presence of naturally occurring heme pigments (as well as hemoglobin due to hemolysis) determined quantitatively by the benzidine reaction.

**113. The bilirubin-binding capacity (BBC) of blood.** Derek Watson, Diane D. Nankville, and Mun H. Own (*Department of Biochemistry, The Royal Womens' Hospital, Carlton N.3, Victoria, Australia*).

**114. The antepartum diagnosis of hemolytic disease by amniotic fluid bilirubin analysis.** A. G. Stewart (*Department of Pediatrics, University of Alberta Hospital, Edmonton, Alberta, Canada*).

The ability to detect accurately the fetus affected with hemolytic disease is important, since death in utero or in the newborn period can be prevented by premature delivery. Analysis of amniotic fluid by means of spectral absorption curves was insensitive, but suggested that the bilirubin content might be a useful diagnostic aid. Accordingly the bilirubin content of amniotic fluid samples obtained from 68 maternity patients at the time of rupture of the membranes has been estimated by a modified Brüchner method and correlated with the newborn infant's clinical condition.

An amniotic fluid bilirubin content of less than 0.035 mg./100 ml. was associated with 36 normal infants and 1 case of mild ABO hemolytic disease. An amniotic fluid bilirubin content over 0.057 mg./100 ml. was associated with 20 abnormal infants. There were 11 fluids with bilirubin contents between 0.035 and 0.057 mg./100 ml.; 5 of these were associated with normal infants, and 6 with infants with hemolytic disease. It is concluded that estimation of the amniotic fluid bilirubin content alone is not a suitable basis for forecasting the health of the newborn infant if it is in the range 0.035–0.057 mg./100 ml. However, if the maternal antibody titre was positive, an amniotic fluid bilirubin content above 0.035 mg./100 ml. was always accompanied by an infant affected with hemolytic disease, whereas below this figure, the infant was unaffected. It appears that a reliable forecast may be made when both the amniotic fluid bilirubin content and the maternal antibody titre are considered.

**115. Studies on the determination of urobilinogen in urine as urobilinogen-aldehyde.** Richard J. Henry, Alberto A. Fernandez, and Sam Berkman (*Bio-Science Laboratories, Los Angeles 25, Calif.*).

In an attempt to complete the standardization of the "quantitative" method for the determination of urobilinogen in urine, recoveries of added urobilin varied erratically between 50 and 80%. Data presented suggest that urobilin is altered in the reduction procedure by the presence of urine and that urobilinogen is oxidized in part to compounds other than urobilin, not rereducible to urobilino-

gen. Improvements in the urobilinogen-aldehyde method for the determination of urobilin and urobilinogen in urine will depend upon separation of these substances from the interfering materials prior to chemical manipulation.

## *SECTION B: CARBOHYDRATES*

**116. Clinical applications of infrared spectroscopy. III. Identification of sugars.** Bernard Klein and Milton Weissman (*Biochemistry Laboratory, Veterans Administration Hospital, Bronx 68, N. Y.*).

Identification of urinary sugars is a frequent clinical biochemical problem. Glucose oxidase indicates only the presence of glucose, and interference by galactose has been reported when impure enzyme preparations are used. The classic specific tests for other sugars when applied directly to urine give colors which are difficult to interpret. Paper and thin-layer chromatographic technics have helped enormously, but reliance is placed on interpretation of $R_f$ values and color reactions, and known markers must be used simultaneously with each determination to serve as reference points.

In this laboratory, a newer approach has been investigated. The ability of the reducing sugars to form insoluble osazones has been utilized to prepare solid derivatives for examination by infrared spectroscopy. The readily prepared derivative is separated, carefully dried, and its infrared spectrum is obtained by the potassium bromide–pressed disk method. The spectrum thus obtained is compared with the spectral patterns given by known sugar osazones. The infrared spectra of the osazones of glucose, galactose, mannose (which forms the hydrazone only under these experimental conditions), lactose, fructose and maltose, arabinose, and xylose are presented. Their similarities and differences are discussed in detail. Interpretation problems related to polymorphism and solute-solvent (matrix) interaction were not encountered.

**117. Enzymatic determination of glucose in urine following rapid removal of inhibitors by ion-exchange resin.** J. E. Logan and D. E. Haight (*Clinical Laboratories, Laboratory of Hygiene, Department of National Health and Welfare, Ottawa, Ontario, Canada*).

Solomon and Johnson (1959) described a method in which a 1:1 mixture of dried anionic and cationic ion-exchange resins were used for the removal of substances present in urine capable of inhibiting the glucose oxidase enzyme system in the determination of glucose. In this laboratory the time required for this adsorption has been reduced from one hour to a few minutes by agitation of the specimens with the resin during this process. A simplification of the separation of the deionized urine from the resin will also be described. Recovery experiments indicate the successful removal of inhibitors, with negligible

loss of glucose. Following resin treatment, the glucose concentration of the urines was determined by a modification of the automated enzyme procedure of Hill and Kessler (1961). Results of the semiquantitative determination of treated and untreated urines using commercial copper reduction tablets and paper strips impregnated with glucose oxidase reagents are compared with the quantitative data obtained by the method above.

**118. Studies with a simplified nitroprusside test for ketone bodies in urine and serum.** Joseph Fraser, Marion C. Fetter, Raymond L. Mast, and Alfred H. Free (*Ames Research Laboratory, Elkhart, Ind.*).

A test has been devised in which a strip of bibulous cellulose is impregnated with the reagents, which consist of sodium nitroprusside, aminoacetic acid, and phosphate buffer to provide a *p*H of approximately 9. These strips, when moistened with urine or serum, develop a purple color in the presence of ketone bodies (acetoacetic acid or acetone), whereas if the sample contains no ketone bodies, no color development occurs. Only 15 sec. are required for the reaction with urine, serum, or plasma. Positive reactions are readily recognized with 10 mg. of acetoacetate per 100 ml. The bibulous reagent strips are relatively stable when stored in a glass bottle containing a desiccant packet. Studies on a series of over 2000 urines from hospital patients and from healthy subjects in comparison with standard procedures indicate that the test has a high degree of specificity and does not give a significant number of false-positive or false-negative results Bromsulfalein in urine may, as with other alkaline nitroprusside tests, give an impression of a positive reaction. Reactivity of the reagent system with serum samples has been investigated and it appears to provide a rapid sensitive tool for recognizing elevation of serum or plasma ketone levels. Data are presented to indicate the utility of simultaneous urine and serum ketone body levels in assessing the severity of ketosis. The simplicity of the test makes it readily adaptable to routine usage in the busy laboratory.

**119. Screening tests for cerebrospinal fluid glucose.** Helen M. Free and Jeanne A. Wasmundt (*Ames Research Laboratory, Elkhart, Ind.*).

Simplified technics have been developed for detection of low CSF glucose by both enzymatic and copper reduction methods. These tests are quite easy to do at the time of the spinal tap and require only minimal amounts of spinal fluid.

With the enzymatic test, one drop of CSF is placed on the glucose test portion of a combination strip test designed for detection of glucose and protein in urine. The glucose test portion contains glucose oxidase and a catalyst-chromogen complex and turns from red to purple in the presence of glucose. Ten seconds after the addition of CSF to the strip, the reaction is matched to the regular 4-block color chart. A negative or light color indicates low CSF

glucose (30 mg./100 ml. or less) whereas a medium or dark color indicates a normal or high CSF glucose (40 mg./100 ml. or more).

With the copper reduction test, 10 drops of CSF and 10 drops of water are placed in a small test tube and a heat-producing copper reagent tablet is added. The tablets contain cupric sulfate, sodium carbonate, citric acid, and sodium hydroxide. After boiling stops, the tube is shaken and, 30 sec. later, compared with the color chart. A blue color indicates low CSF glucose, green usually indicates normal, and brown usually indicates elevated CSF glucose.

Data are presented to indicate that the tests are reliable screening methods particularly suited for detection of low CSF glucose.

**120. A study of the Momose fluorometric determination of blood glucose.** Bernadette B. Bourne (*Departments of Neurology and of Biochemistry, University of Miami, Miami, Fla.*).

The fluorometric assay of blood glucose by the method of Momose and Ohkura (*Talanta 3*:151, 1959) using 5-hydroxytetralone was adapted to the Aminco-Bowman spectrophotofluorometer, with a resulting increase in sensitivity. Activation and fluorescence wavelengths used were 470 and 550 mμ, respectively, the uncorrected maxima.

The findings of Momose that sucrose, cellulose, mannose, galactose, fructose, and glyceraldehyde react with the reagent to give a like product were confirmed. In addition, interference due to ascorbic acid, citric acid, pyruvic acid, lactic acid, and heparin could not be shown at levels normally present in fasting blood specimens.

Oxalated blood specimens (random individual samples and glucose tolerance specimens) containing sodium fluoride, were obtained from the clinical chemistry laboratory of Jackson Memorial Hospital, where the Somogyi filtrates had been automatically analyzed for glucose by the iron reduction method. New Somogyi filtrates were prepared and analyzed in our laboratory by the Nelson copper reduction macro method. The ultramicro method of Momose was used simultaneously on the TCA supernatants. Good agreement with the Hagedorn method was previously reported by Momose on a limited number of normal specimens. Comparison of results of these methods appears to warrant further study for adaptation to semi- or fully automated fluorometric procedures and to further ultramicro modification for pediatric application in which 5 μl or less of heel- or finger-prick blood would be adequate.

**121. Study of some enzymes of hexose monophosphate shunt in diabetics.** J. A. Lopes do Rosário (*Laboratório de Química Fisiológica, Faculdade de Medicina, Lisbon, Portugal*).

The study of the glucose-6-phosphatase activity in liver biopsies of patients with diabetes proved the increase of that activity (in relation to normal sub-

jects) and the return to normality after treatment with insulin or hypoglycemic sulphamides. Supposing that an equilibrium exists among all phosphorilated monosaccharides (Bonsignore), each phosphorylation should effect the others.

In relation to many papers published about glucose-6-phosphate dehydrogenase in situations where this enzyme is decreased in activity or even absent (favism, medicinal poisonings, etc.), other enzyme activities of the hexose monophosphate shunt (ketopentose phosphate isomerase, transketolase, transaldolase) were studied and the results obtained in plasma and in red cell hemolysates of diabetic patients not treated or compensated are described.

The results obtained show a clear decrease in activity of the enzymes studied.

**122. The concentration of glucose and other reducing substances in different organs of fed and fasting rats.** J. Krawczynski, R. Vrba and H. S. Bachelard (*Department of Laboratory Diagnostic, Postgraduate Medical School in Warsaw, Poland, and the Neuropsychiatric Research Unit, Medical Research Council, Carshalton, Surrey, U. K.*).

Three groups of organs were investigated:

1. The organs in which the level of glucose and other reducing substances after 24 hr. of fasting remains unchanged (brain, cerebellum)
2. The organs in which the concentration of total reducing substances in fasting animals decreases significantly but without change of the ratio of glucose to other reducing substances (kidney, spleen, heart, blood)
3. The organs in which the concentration of glucose decreases in fasting animals, with only slight decrease of other reducing substances (liver, skeletal muscle)

The "true glucose" was estimated with the yeast fermentation method of Holt, modified by the authors.

The practical and theoretical significances of the observed changes in concentration of glucose and other reducing substances after 24-hr. fasting are discussed.

**123. The pentose metabolic pathway in neoplastic disease.** Y. S. Brownstone (*Department of Pathological Chemistry, U.W.O., London, Canada*).

Serum samples were obtained from normal individuals and patients in a variety of pathologic and physiologic conditions. The activity of the enzymes of the pentose phosphate metabolic pathway (PPMP) in these sera was assayed by incubation in a medium containing Tris buffer (*p*H 7.5) and ribose-5-phosphate substrate, and maintained at 37°. Both dialyzed and undialyzed serum was used as a source of enzymes. The effects on enzyme activity of enzyme concentration, addition of co-factors (magnesium and thiamine pyrophosphate),

and the addition of various inhibitors were investigated. Enzymatic products were assayed colorimetrically (Brownstone and Denstedt. *Can. J. Biochem. Physiol. 39*:533, 1961) and separated by paper chromatography and ion-exchange chromatography. Studies have been carried out to relate the serum enzymes with the tissue of origin. Enzyme activity was shown to be elevated in patients with neoplastic disease. The requirement for co-factors by the enzymes in these sera differs from those of normal tissue investigated. The course of the disease was followed with serial determination of enzyme activity and the effect of therapy noted.

**124. The glycogen content of muscle tissue obtained by needle biopsy on normal and diabetic man.** Jonas Bergström and Eric Hultman (*The Central Clinical Laboratory, St Erik's Hospital, Stockholm, Sweden*).

Muscle samples from the quadriceps femoris were obtained by needle biopsy, and the trichloroacetic acid extractable glycogen was determined by the orthotoluidine method after ethanol precipitation and acid hydrolysis.

The glycogen content of muscle was studied in normal man under different conditions. The effect of carbohydrate infusions, with and without insulin, and the effect of fasting and of muscular work on the glycogen content was investigated.

The muscle-glycogen level in patients with juvenile diabetes before and during insulin treatment is also reported.

**125. Technic and interpretation of continuous intravenous glucose recordings.** Einhart Kawerau and S. J. Surtus (*Department of Chemical Pathology, St. James Hospital, Sarsfeld Road, Balham S.W. 12, England*).

A technic for continuous intravenous glucose recording with the AutoAnalyzer has been devised. The resulting record is called the glucogram.

A suitably modified alkaline ferricyanide reduction method as well as adaptations of the current glucose oxidase methods have been used as the means of assay.

Some basic physiological responses have been studied, such as the effect of exercise, of a variety of meals, of sleep, and of leucine, insulin, and glucagon. Drug effects that have been studied include those of orally administered sulfonamide, salicylate, and alcohol (ethanol), and intravenously injected chlorpropamide, and tolbutamide.

The glucogram has been used in the clinical assessment of diabetes in over 60 patients. More than 40 have been tested by a double technic consisting of an intravenous glucose injection followed by an intravenous injection of a sulfonylurea after the fasting blood sugar level had been regained. From these glucograms the diminution rate of the blood sugar, in percentage per minute (K-value), was calculated and interpreted together with the sulfonylurea re-

sponse. It has become clear that both tests must be interpreted independently, as the mode of operation apparently differs.

The clinical implications is discussed. The glucograms indicate that the standard procedure for arriving at the K-value, as suggesting by Lundbaek, can be simplified. A method for calculating K from two blood sugar values taken at 30-min. intervals is described.

### *SECTION C: PROTEINS AND GLYCOPROTEINS*

**126. The fluorometric determination of albumin in cerebrospinal fluid and serum.** Alex Kaplan and Murray A. Johnstone (*Department of Biochemistry, University of Washington, Seattle 5, Wash.*).

Ultramicromethods for the determination of serum albumin by fluorescence with specific dyes have been adapted for the measurement of the albumin concentration of cerebrospinal fluid. Results obtained with the use of 1-anilino-naphthalene-8-sulphonic acid (Rees, V. H., *et al. J. Clin. Path. 7*:336, 1954) and with Vasoflavine (Betheil, J. J. *Anal. Chem. 32*:560 1960) are discussed. The concentrations of albumin found in a variety of cerebrospinal fluids and sera by fluorometric methods are compared with those obtained by electrophoresis on cellulose acetate. The volume of normal cerebrospinal fluid required for an analysis varies from 0.05 to 0.2 ml., depending upon the dye selected and the sensitivity of the fluorometer.

**127. The detection of abnormal proteins in plasma and serum by viscometry.** John Harkness and R. B. Whittington (*Department of Clinical Pathology, Taunton and Somerset Hospital, Musgrove Park Branch, Taunton, England*).

A new instrument for the accurate automatic estimation, at about one test per minute, of the viscosity of plasma and serum is described.

The viscosity of plasma and serum depends upon the concentration of the proteins, especially the fractions with high molecular weight. Physiological stimuli (apart from pregnancy) do not cause marked changes in these large components, and in normal subjects the plasma and serum viscosity values fall within narrow ranges.

In clinical biochemistry, the measurement of the viscosity of plasma and serum is a sensitive and rapid method of detecting the certain presence or probable absence of abnormal amounts of fibrinogen and globulins. A variation from the usual pattern of viscosity changes relative to total proteins can indicate the presence of macroglobulins.

In clinical pathology, as a corollary of that above, the viscosity measurement can be used in place of the erythrocyte sedimentation rate as a guide to the presence and severity of a reaction to infection, malignancy, etc.

**128. Serum alpha-2 macroglobulin levels in health and disease.** M. S. deWolfe (*Research Institute, The Hospital for Sick Children, Toronto, Ontario, Canada*).

No physiological role has been assigned to alpha-2 macroglobulin in human serum, and very little is known about its behavior in disease. Little quantitative data is available as to levels of this macroglobulin either in health or disease. Using a modification of the quantitative precipitin technic of Kabat and Mayer, alpha-2 macroglobulin levels have been studied in 11 healthy adults, 28 healthy children, 33 children with nephrosis, and 25 children suffering from a variety of pathologic conditions in which alterations in serum proteins might be anticipated. In health, serum alpha-2 macroglobulin levels have been found to be high at birth and to remain high throughout childhood. In healthy adults, the level has fallen to less than half that found in healthy children, female adults showing a significantly higher level than male adults. Male children have alpha-2 macroglobulin levels equal to or slightly greater than female children of comparable age. Striking elevations of this serum protein were found in nephrosis, the alpha-2 macroglobulin in some cases representing over 50% of the total serum protein. Lesser increases were observed in chronic glomerulonephritis, but normal levels were found in other types of renal disease studied.

**129. Starch gel electrophoresis patterns of serum proteins in cystic fibrosis of the pancreas.** Eleanor R. Harpur, Anne H. Murdoch, and Mimi M. Belmonte (*Departments of Biochemistry and Metabolism, the Montreal Children's Hospital, Montreal, P.Q., Canada*).

Initial studies of serum proteins using the Smithies technic of starch gel electrophoresis indicated the presence of a specific alteration in the patterns of cystic fibrosis patients, their parents, and some of their sibs. The post $\beta_1$ fraction is decreased in concentration while its mobility is normal. The post $\beta_2$ fraction on the other hand has decreased mobility, but its concentration is occasionally increased. These alterations are found to occur with statistically significant frequency. It has been suggested that these specific protein fractions may be altered by antigenic stimulation.

**130. Serum polysaccharides in transplantable and induced tumors of mice.** Yasin Abd El-Ghaffar (*Abbasian Faculty of Medicine, Cairo, U.A.R.*).

Sarcoma I80 and methyl cholanthrene-induced sarcoma were used as models for transplantable and induced tumors, respectively. The serum polysaccharides (S.P.) were estimated by the tryptophan method.

In mice bearing sarcoma I80 a progressive rise in level of S.P. took place during the first week after transplantation. In the second week the level was nearly stationary, while in the third week it continued to fall, until it reached normal or subnormal values at the time of the death of the animal.

In mice receiving methyl cholanthrene subcutaneously a preliminary fall in the level of S.P. was observed in the first 2 weeks after injection, followed by a gradual and slow rise until the appearance of the tumor. Then the level progressively fell as the tumor increased in size.

Possible interpretations of these findings are discussed.

**131. Action of α-lipoic acid on ceruloplasmin and transferrin in several illnesses.** S. F. Gomes da Costa, J. A. Filipe da Silva, and Felisbela E. S. Maldonado (*Laboratory of Physiological Chemistry, Medical Faculty, Lisbon, Portugal*).

The action of α-lipoic acid on ceruloplasmin, transferrin, copper and iron of blood, and urine in hepatic cirrhosis, hepatitis, pregnancy, acute glomerulonephritis, and polyneuritis was tested. Comparative study was begun of thiamin and cobalamin. It was concluded that α-lipoic acid does not affect only labile connections of copper with blood protides, but also ceruloplasmin, causing its decrease in these illnesses (the opposite is observed in hepatolenticular degeneracy). By and large, cupremia and cupruria increase. The action of thiamin is parallel except in glomerulonephritis. In the same cases α-lipoic acid practically always caused increase of transferrin (except in that illness) and of sideruria (with the same exception). In relation to iron metabolism no parallelism between the actions of α-lipoic acid and thiamin was ascertained, as in relation to copper, and also more divergencies through the action of cobalamin were verified in relation to iron and copper metabolism.

The variation caused by α-lipoic acid on ceruloplasmin and transferrin was inverse, while generally the cupruria and sideruria increased. α-Lipoic acid act in the same way on labile or strong protidic copper connections but not in relation to iron. The variations brought about by α-lipoic acid in the metabolism of these two metals, is conditioned by the illnesses' chemical pathology.

**132. Polarographic determination of fucose in serum proteins and protein-containing tissue extracts.** Z. Stary, A. H. Wardi, and D. L. Turner (*Warren State Hospital, Warren, Pa.*).

The method includes the precipitation of the serum proteins with alcohol, the liberation of the fucose from the precipitate by partial hydrolysis with acid, and the removal of amino acids and peptides from the hydrolyzate by passing through an IR-120 ion-exchange column. The eluate is concentrated and treated with an excess of 2.0% periodic acid at room temperature for 1 hr. This treatment oxidizes the fucose, and an equivalent amount of acetaldehyde is formed; monosaccharides which do not contain a methyl group give formaldehyde in this treatment. The mixture is then neutralized with LiOH and the periodate is removed by passing through a columnu of IR-400. The eluate is cooled to 14° and polarographed between 1.5–2.0 v in a N/10 LiOH solution. The amount of fucose is determined by comparing the wave height of the sample with that of a

similarly treated fucose standard. In preliminary experiments, the conditions were studied under which fucose can be liberated quantitatively from the prosthetic carbohydrate groups of glycoproteins. The fucose content of tissue mucoproteins can be determined by a similar procedure after extraction of the mucoproteins with slightly alkaline KCl solutions or by direct analysis of solutions obtained by partial acid hydrolysis of the tissues.

**133. The effects of age and sex on the electrophoretic fractions of the serum glycoproteins.** R. H. Pearce, E. M. Watson, R. Stodolski, J. M. Mathieson, and J. J. Theoret (*Department of Pathological Chemistry, Faculty of Medicine, University of Western Ontario, London, Canada, and Department of Pathology, Faculty of Medicine, University of British Columbia, Vancouver, Canada*).

Specimens of serum were collected from 198 clinically healthy persons chosen to provide approximately 10 males and 10 females in each decade up to the tenth. After electrophoretic separation using the Spinco method B, the strips were stained by the periodic acid-Schiff reaction according to Koiw and Gronwall (*Scand. J. Clin. Lab. Invest. 4*:244, 1952) and scanned on the Analytrol integrating densitometer at 550 mμ. Total glycoprotein was estimated on an ethanol precipitate by the tryptophan-borosulphuric acid reaction (Badin, Jackson, and Schubert. *Proc. Soc. Exp. Biol. Med. 84*:288, 1953).

The analytical error and normal values for the method were estimated; those for males did not differ from those for females. The means and standard deviations for groups showing statistically-significant differences ($P < 0.05$) were, in milligrams per milliliter of serum: total under 70 yr., 148 ± 19; total over 70 yr., 164 ± 20; albumin, 23.1 ± 6.6; alpha-1, 12.5 ± 4.3; alpha-2 under 60 yr., 47.4 ± 12.1; alpha-2 over 60 yr., 59.1 ± 16.0; beta under 10 yr., 27.2 ± 6.6; beta 10–90 yr., 38.4 ± 8.2; beta over 90 yr., 54.0 ± 17.7; gamma, 26.4 ± 7.1. The results were compared with those obtained by other published procedures.

**134. Biochemical studies on mucopolysaccharides in synovial fluid.** H. Greiling (*Rheumaforschungsinstitut beim, Landesbad, Aachen, Netherlands*).

**135. Studies on serum proteins and lipoproteins in normal Egyptians and in patients with medical kidney diseases.** Ezzat Abdel Wahhab (*Clinical Pathology Department, Faculty of Medicine, Ein Shams University, Cairo, U. A. R.*).

It is known that lipids are present in blood as lipoprotein complexes, the unesterified fatty acids being bound to serum albumin and various proportions of cholesterol, phospholipids, and triglycerides bound to serum globulins.

Various methods for separation and estimation of serum proteins and lipoproteins have been developed depending on differences in their physical, bio-

chemical, and immunological properties. Of these methods, zone electrophoresis on filter paper with subsequent staining of paper with protein or lipid stains proved to be very valuable both for clinical and research work.

This achievement stimulated many workers to investigate the protein and lipoprotein pattern in physiological and pathological conditions and of the effect of these changes, when present, on the course and prognosis of the disease.

It was hoped that studying the lipid changes that accompany medical kidney diseases and the nephrotic syndrome would lead to a better understanding of the pathogenesis of these diseases.

Different lipid stains were tried. Of these, fat red 7 B (Gurr) proved to be superior to other dyes by virtue of the fact that it gives distinct lipid zones and a very clear background. The estimation of the different lipoprotein fractions was done later by scanning and elution.

Estimation of the protein and lipoprotein pattern in 50 normal Egyptians of both sexes in the second, third and fourth decade was done in order to furnish a basis for comparing the pattern obtained in diseased cases.

Values obtained for normals are presented.

The investigation of the protein and lipoprotein pattern in medical kidney diseases is still in progress.

**136. Effects of excremental odor on growth-promoting substances.** Toyoji Yoritaka, Masao Fujimoto, and Ikuo Muta (*Department of Biochemistry, School of Medicine, Nagasaki University, Japan*). Read by title.

The inferiority of the Japanese to Americans or Europeans in constitution and stamina may be due not only to differences in food and modes of life, but also to the Japanese method of feeding of infants in the nursing stage. In Japan nursing infants spend considerable time carried on their mothers' backs and are sometimes left all night without change of diapers. Therefore they often inhale the odor of their own feces and urine for long periods.

This excremental odor penetrates blood through the nose, trachea. and lung. The effects of the odor on growth-promoting substances in blood were investigated in model experiments. Excremental odor was passed through solutions of a certain concentration of amino acids, vitamins, and hormone which are essential for growth, and the decreases for 30, 60, 90, and 120 min. were measured. It is clear from the data gathered that unknown substances with odor destroy to some degree the growth-promoting compounds except vitamin $B_1$.

The results indicated that diapers should always be kept clean so that infants may be prevented from inhaling their own excremental odor as much as possible.

# BOOK REVIEWS

**Atlas of Histopathology of the Skin (ed. 2).** G. H. Percival, George L. Montgomery, and T. C. Dodds. The Williams and Wilkins Company, Baltimore, 1962, $24.95.

The outstanding feature which distinguishes this book from other atlases of dermatopathology is its 525 beautiful, full-color microphotographs. Important neoplastic, inflammatory lesions, and reactions to injury, plus degenerative changes are covered through low-, medium-, and high-power magnifications. Where indicated, Verhoeff, Van Giessen, Weigeret's, polychrome methylene blue, iron hematoxylin, Sudan, Dobell's, Prussian blue, and even bacteriological stains are presented.

The organization is excellent. A glossary and 24-page description and illustration of normal skin and appendages will be invaluable to students, house staff, and practitioners. There are even fine illustrations of common skin parasites.

No gross illustrations of clinical cases are included, but the concise descriptions accompanying each lesion covers clinical findings and classifications.

Some of the low-power illustrations are not clearly diagnostic, but almost all of the higher magnifications are faithful in their clarity of nuclear, cytoplasmic, and color details.

The index is complete and well organized. There is no bibliography, as this is an atlas, not a textbook, although 485 pages belies this.

Exclamations of amazement and praise at the beautiful color reproductions was the unanimous reaction of pathologists to whom this atlas was circulated. It is a must for all teaching departments of dermatology and pathology.

*Metropolitan Hospital*
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*Pathologist-in-Charge*

# The Effect of Chemotherapeutic Agents upon the Metabolism of Intact Human Cancer Cells

## An In Vitro Technic for Cell Sensitivity

*Bernard J. Katchman, Robert E. Zipf, and James P. F. Murphy*

**The effect of certain chemotherapeutic agents upon the metabolism of human ascites and leukemic leukocytes was investigated. Human leukemic blast cells show 2–3 times the respiratory activity of "chronic" cells. From a study of the effects of eight chemotherapeutic drugs upon the respiration of human leukemic leukocytes and human ascites cells, relative index of cell sensitivity was obtained.**

**The alkaloid, vinblastine sulfate, and the alkylating agents, triethylenethiophosphoramide and nitrogen mustard, were found to be more toxic to human cancer cells than the antimetabolites, 6-mercaptopurine, 8-azaguanine, aminopterin, diazouracil, or prednisone. Stimulation of aerobic glycolysis was found for all chemicals except aminopterin (no effect) and nitrogen mustard (inhibition).**

**No correlation between stimulation of lactic acid production and inhibition of respiration was found. Stimulation of lactic acid production could be produced under conditions where respiration was unchanged. Only nitrogen mustard differed in that inhibition of respiration always accompanied inhibition of lactic acid production.**

**Most chemotherapeutic agents tested are Pasteur effect inhibitors.**

**An unusual stimulation of glucose disappearance to products unknown as yet was caused by 6-mercaptopurine in all cells tested. The potential usefulness of a cell sensitivity test in clinical chemotherapy is discussed.**

FOR MORE THAN a decade there has been intensive activity toward the development of an armamentarium of agents for the chemotherapy of

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cancer (*1*). These chemical agents have been classified, in functional terms, as alkylating agents or antimetabolites.

The term alkylating agent, which is meaningful nomenclature to the organic chemist, remains for the biologist a *non sequitur*. The term antimetabolite is meaningful to the biochemist; implicit in this nomenclature is metabolic specificity of action (*2*). The point of this discussion is not merely semantics, but a recognition that the descriptive nomenclature of therapeutic drugs is based upon arbitrary criteria which in no way convey the entire spectrum of effects which may be elicited in mammalian cells by these chemicals.

It has been reported by Laszlo *et al.* (*3*) that the purine antagonist, 6-mercaptopurine, considered to be primarily an inhibitor of nucleic acid synthesis, has notable effects upon the aerobic glycolysis of human acute leukemic leukocytes.

The documentation of the sensitivity of cancer cells to chemotherapeutic agents is not only of value for the development of new drugs, but may be helpful in their clinical application in terms of toxic effects to the patient, choice of drug, and perhaps a better understanding of the reason or mechanism of drug resistance exhibited by the patient with cancer.

In this report we present the results obtained from studies on the effects of chemotherapeutic agents upon the metabolism of human cancer cells and an appraisal of the response of the cells as a routine test in the chemical control of cancer. Also it is suggested that a neoplastic cell may respond in relation to the type and concentration of its chemotherapeutic environment. Parallel studies using a transplantable animal chloromyeloid leukemia (Sprague-Dawley rat) will be reported later.

## Materials and Methods

### Materials

*Aminopterin: 4-aminopteroylglutamic acid* (Lederle) Concentration, 10 mg./ml. (w/v). Dissolve in 10% NaOH; back-titrate with 0.1N HCl to *p*H 10; then adjust to volume with Krebs-Ringer phosphate (KRP) buffer solution.

*Nitrogen mustard: Methyl-bis(β-choloroethyl)amine hydrochloride* (Merck & Co.) Concentration, 10 mg./ml. (w/v). Dissolve in (KRP) buffer solution and adjust to volume.

*Meticorten: prednisone, 5 mg. in Meticorten tablet* (Schering) Con-

centration, 5 mg./ml. (w/v). Suspend tablets in (KRP) buffer solution.

*6-Mercaptopurine* (Nutritional Biochemical Corporation) Concentration, 10 mg./ml. (w/v). Dissolve in 10% NaOH; back-titrate with 0.1N HCl to *p*H 10; adjust to volume with (KRP) buffer solution.

*Thio-TEPA: N,N',N'' triethylenethiophosphoramide* (Lederle) Concentration, 5 mg./ml. (w/v). Dissolve in (KRP) buffer solution and adjust to volume with buffer solution.

*8-Azaguanine* (Nutritional Biochemical Corporation) Concentration, 10 mg./ml. (w/v). Dissolve in 10% NaOH; back-titrate with 0.1N HCl to *p*H 10. Adjust to volume with (KRP) buffer solution.

*Velban: vinblastine sulfate* (Eli Lilly & Co.) Concentration, 2.5 mg./ml. (w/v). Dissolve in (KRP) buffer solution and adjust to volume with buffer solution.

*Diazouracil* (Nutritional Biochemical Corporation) Concentration, 10 mg./ml. (w/v). Dissolve in 10% NaOH; back-titrate with 0.1N HCl to *p*H 10. Adjust to volume with (KRP) buffer solution.

*Fibrinogen: Fraction I, from bovine plasma* (Armour & Co.) Concentration, 31 mg./ml. (w/v). Aqueous solution made up fresh before use.

*Monocote E: Arquad 2C* (Armour Laboratories)*

*Siliclad: water soluble silicone* (Clay Adams)†

*Krebs-Ringer Phosphate Buffer Solution* According to the method of Umbreit (*4*).

*Heparin Solution: Sodium heparin 1 mg./ml. (w/v)* (Upjohn Co.)

*Glucose standard solution, 0.1M, 18 gm./l. (w/v)* (Merck Co.)

*Potassium hydroxide* 20% (w/v) aqueous solution

## Methods

The method of Minor and Burnett (*5*), with slight modification, was used for *preparation of the cell suspension for Warburg manometric study.* Blood is withdrawn (30 ml.) in a silicone-coated syringe and needle wetted with heparin and, after removal of the needle, transferred to 20-ml. test tubes containing 0.6 mg. heparin solution per 15 ml. blood. To each tube of blood (approximately 30 mg.) is added 1.9 ml. of bovine fibrinogen Fraction I solution. They are then gently mixed by inversion. The tubes are set at a 60° angle and incu-

*Used to coat needles.

†Used to coat all glass surfaces with which leukocytes come into contact.

bated at 37° for 30 to 45 min. The supernatant is carefully aspirated while the tube is still held in an inclined position. The leukocyte count is determined in duplicate by the standard hemacytometer technic, and aliquots to provide between 100 and 200 $\times$ $10^6$ cells are taken for study.

Respiratory oxygen uptake was determined by the method outlined byUmbreit (*6*). For the measurement of oxygen uptake the following additions are made to Warburg flasks: in the center well, 0.2 ml. of 20% KOH; in the main flask, 0.2 ml. of 0.1M glucose, aliquot of cell suspension in plasma, aliquot of chemical from stock solutions as outlined above, and buffer to a final volume of 3.2 ml. Flasks are attached to manometers and manometric readings are begun. Manometric experiments were carried for periods of 1–3 hr., depending upon the respiratory activity of the cells.

The most convenient procedure is to add cells, chemical, and glucose to the flasks and after equilibration in the water bath to take manometric readings and subsequently, at the end of the experiment, to obtain lactic acid and glucose concentrations. With this type of experiment one may compare the results of the control cells with those of the treated cells as percentage of control. However, when more refined data are required, such as rate of production of lactic acid or effect of time of contact of chemical upon respiration, then it is more convenient to tip in the chemical from the side arm at zero time. The latter procedure is recommended as a standard procedure not only for convenience but because it provides the best technic for evaluation of chemical sensitivity, as will be seen below.

At the end of each experiment, 2.0 ml. of 10% trichloroacetic acid is added to each flask to stop the reaction. After centrifugation the supernatants are analyzed for lactic acid and glucose. Lactic acid was determined by the chemical method of Barker and Summerson (*7*); glucose by the enzymatic method of Keston as modified by McCombs and Yoshuk (*8*).

For study of ascites cells, cell suspensions were obtained by centrifugation of ascitic fluid from which the cellular material was washed with 0.9% NaCl and resuspended in 0.9% NaCl. Because of clumping of the cellular material, reproducible cell counts could not be obtained and therefore a "hematocrit" reading of the centrifuged cell suspension was used as a measure of cell volume.

Studies were made on samples obtained from patients at this hospital having a cell count in the peripheral blood or ascites fluid high

enough to enable us to study cell sensitivity regardless of previous history with respect to therapy or duration of the diseased state.

## Results

The parameters measured were: (1) oxygen uptake as a measure of the respiratory activity, (2) aerobic formation of lactic acid as a measure of aerobic glycolysis, and (3) glucose disappearance or utilization as a measure of the total carbohydrate metabolic potential.

### In Vitro Respiration of Human Cancer Cells

The respiration or oxygen uptake may be measured in a Warburg respirometer. Figure 1 is an example of human tumor cell activity. Under optimum conditions, the rate of oxygen uptake is linear with time; this is a condition of zero-order kinetics. Any condition which alters the rate of oxygen uptake but not the kinetics of the reaction will change the slope of the activity curve but not its shape. It can be seen in Fig. 1 that the effect of glucose added to tumor cells from pleural fluid of a patient with adenocarcinoma primary in the ovary is a depression of oxygen uptake to 54% of the rate found without added glucose. In another patient with the same diagnosis the depression caused by added glucose was to 78% of the value without glucose. This phenomenon is found in all animal tumors; it is called the Crabtree effect (*9*).

It should be noted that human leukemic leukocytes show the Crabtree effect, but not to any degree comparable to that seen with ascites cells. The measurement of the respiration of leukocytes is usually carried out in homologous plasma and the Crabtree effect was found to be only about 10%. The mechanism of the Crabtree effect is still obscure

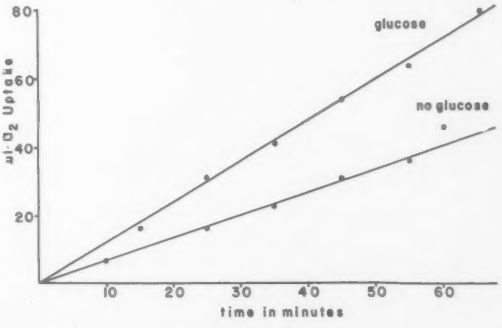


**Fig. 1.** Human tumor cell respiratory activity and the Crabtree effect. Upper curve obtained in absence of glucose; lower curve, in presence of 3.6 mg. glucose.

(*10*). The reason that human leukemic leukocytes do not exhibit this effect to as large a degree as ascites cells may be due to the fact that leukemic leukocytes are derived from a more physiological environment (homologous plasma) than are ascites cells.

The respiratory activity of human leukemic leukocytes is tabulated in Table 1. The data for the ascites cells are omitted, as accurate cell counts could not be obtained. However, for these 2 cases of adenocarcinoma primary in the ovary, the data were normalized to a "hematocrit" unit and upon that basis good agreement was found between the two samples.

The point of interest in Table 1 is the ready distinction between acute (high activity) and chronic states (low activity). Each value represents a sample drawn at least one day apart from another. The relatively constant value obtained for each patient is a good indication of the reliability with which these calculations may be made. In the entries under Case 11 in the table, one of the three values was obtained one year later than the others. It is noteworthy that the metabolic

**Table 1.** Respiratory Activity of Human Leukemic Leukocytes

| *Case No.* | | *Activity* ($\mu l.$ $O_2/10^6$ *cell/hr.*) | *Diagnosis* |
|---|---|---|---|
| 1 | | 0.24 | Chronic lymphatic leukemia |
| | | 0.26 | |
| 2 | | 0.23 | Chronic lymphatic leukemia |
| 3 | | 0.27 | Chronic lymphatic leukemia |
| 4 | | 0.21 | Chronic lymphatic leukemia |
| 5 | | 0.18 | Chronic lymphatic leukemia |
| | | 0.20 | |
| 6 | | 0.27 | Subacute lymphatic leukemia |
| | | 0.27 | |
| | | 0.21 | |
| | | 0.22 | |
| 7 | | 0.28 | Chronic myelogenous leukemia |
| | | 0.32 | |
| 8 | | 0.29 | Leukemoid reaction due to reticulum cell sarcoma |
| | | 0.29 | |
| 9 | | 0.43 | Stem cell leukemia |
| | | 0.48 | |
| | | 0.45 | |
| 10 | | 0.57 | Acute myelogenous leukemia |
| 11 | (2-28-61) | 0.37 | Acute myelogenous leukemia |
| | (3- 3-61) | 0.43 | |
| | (2- 2-62) | 0.41 | |

activity of "blast cells" is nearly twice that of the more mature cells as seen in chronic leukemia.

### Effect of Chemicals upon $O_2$ Uptake of Human Cancer Cells in Vitro

The evaluation of the effect of chemicals upon cellular respiration is not always straightforward. Certain factors which do not always obtain serve to complicate the kinetics of reaction. These factors may be related to the cell type, physiologic state of the cell, type and/or concentration of chemical used, incubation time or contact time of cell and chemical, and finally the effect of added glucose. The effects of some of these factors will be demonstrated below; however, too few cases have been studied to establish definitely that such factors are responsible for variations when all are not controlled.

With the exceptions or qualifications above in mind, it is still possible to demonstrate under the experimental conditions employed, that in the presence of certain added chemicals and glucose, cancer cells show zero-order kinetics in oxygen uptake rate. This type of reaction is shown in Fig. 2, where the inhibition of respiration by diazouracil of cells from a patient with chronic lymphatic leukemia is demonstrated. From the slopes of these two curves it is a simple matter to determine inhibition. The effect upon cellular respiration is recorded as percentage of control oxygen uptake and the chemical concentrations which produce the effects are normalized to micrograms per $10^6$ cells. When percentage control oxygen uptake is plotted against chemical concentration on semi-log paper, a straight-line relationship is found which intersects the 100% activity point at zero concentration. The slope of the line then represents the sensitivity of the cell to chemi-

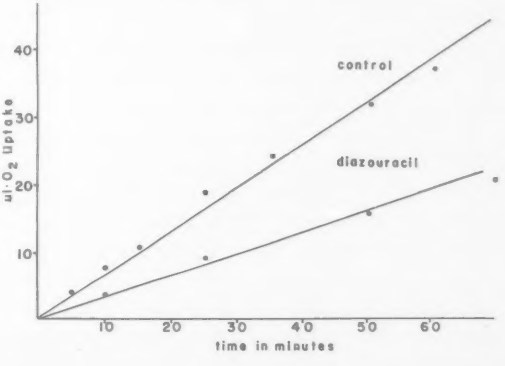


**Fig. 2.** Inhibition of respiration of human leukemic leukocytes by diazouracil. Upper curve represents activity of 125 × $10^6$ cells; lower curve, activity of 125 × $10^6$ cells in presence of 4.5 mg. diazouracil.

cal; for comparison purposes the concentration of chemical which produces 50% inhibition may be used.

This type of treatment of the data is illustrated in Fig. 3. The cells were obtained from the pleural fluid of 2 patients with adenocarcinoma

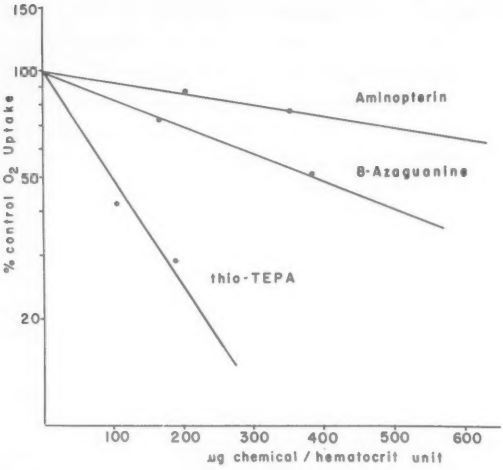


**Fig. 3.** Determination of sensitivity of human tumor cells to chemotherapeutic drugs. Plot of percentage activity versus normalized chemical concentration.

primary in the ovary. The percentage of control is plotted on semi-log scale against an arbitrary normalized chemical concentration per unit of cell activity. Even though only one experimental point per individual per chemical is plotted, the data show good fit to the curves. It is obvious that the cancer cells in this instance are most sensitive to thio-TEPA (triethylenethiophosphoramide) and least sensitive to aminopterin.

At this point, it is germane to present some data in the case where the chemical produces kinetic effects that require analysis of the activity time curves before inhibition can be quantitated.

Figure 4 shows the control rate curve and that for the thio-TEPA–treated cells obtained from a patient with chronic myelogenous leukemia. (Note that the thio-TEPA curve is made up of two zero-order parts.) After 15 min. the zero-order inhibition curve is apparent.

Figure 5 shows the rate curves for both control and thio-TEPA–treated cells obtained from a patient with stem cell leukemia (acute). (Note that the thio-TEPA curve is made up of two parts.) Initially, there is stimulation of oxygen uptake well above that for the control.

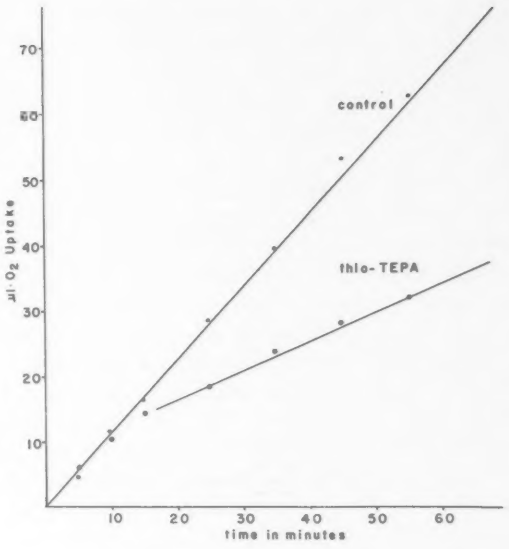


**Fig. 4.** Effect of thio-TEPA on human leukemic leukocytes. Upper curve represents activity of $203 \times 10^6$ cells; lower curve, activity of $203 \times 10^6$ cells in presence of 2.5 mg. thio-TEPA.

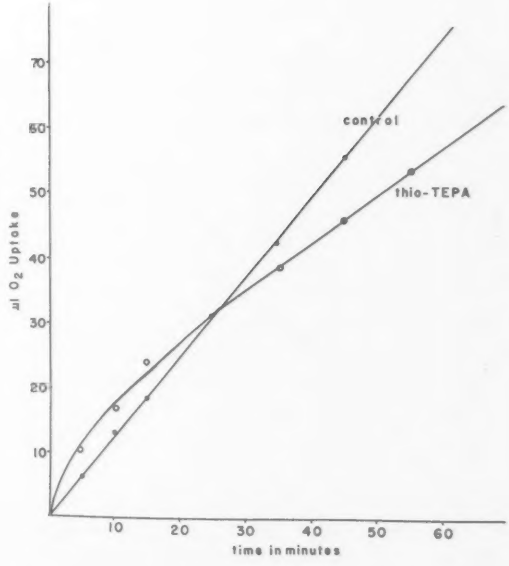


**Fig. 5.** Effect of thio-TEPA on human leukemic leukocytes. Upper curve represents activity of $168 \times 10^6$ cells; lower curve, activity of $168 \times 10^6$ cells in presence of 3.0 mg. thio-TEPA.

At 25 min., the thio-TEPA curve intersects the control curve and subsequently a zero-order inhibition curve is obtained.

The point to be made about the curves in Fig. 4 and 5 is that the true inhibition would be underestimated if correction were not made for changing kinetic patterns. For example, in Fig. 5, if the total oxygen uptake for 50 min. of the control and treated cells are compared, the percentage activity calculated is equal to 80%. However, if the slopes of the two curves are compared after 25 min., the ratios of activity of cells treated to control cells is equal to 53%.

Figure 6 shows the control rate curve and that for thio-TEPA–treated cells obtained from a patient with chronic lymphatic leukemia. In this case, note that the oxygen uptake is above the control rate and also that this curve is logarithmic in nature. This is also represented in Fig. 7 where the percentage of control activity is plotted against time on semi-log paper. The straight line which is thus obtained indicates a first-order reaction for the kinetics of inhibition.

It is obvious that a chemical which produces inhibition without affecting the order of the reaction with respect to the control reaction can readily be evaluated with respect to cell sensitivity; the concentra-

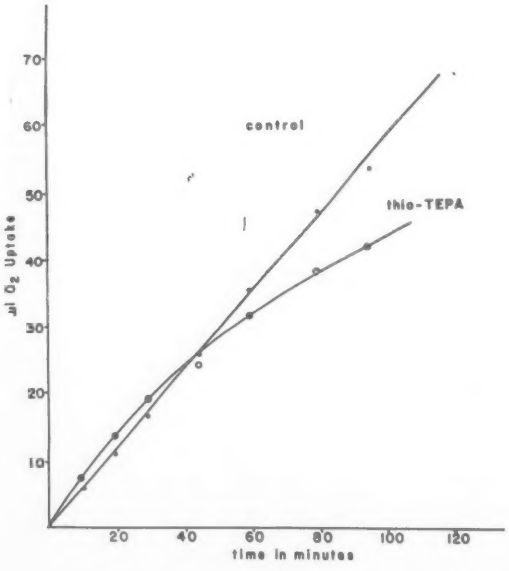


**Fig. 6.** Effect of thio-TEPA on human leukemic leukocytes. Upper curve represents activity of $207 \times 10^6$ cells; lower curve, activity of $207 \times 10^6$ cells in presence of 5.0 mg. thio-TEPA.

tion that causes 50% inhibition may be readily ascertained. This is not the case when the rate of inhibition is logarithmic for, under these circumstances, both the chemical concentration and the time of contact are variables. Thus, for each concentration there is a separate time-

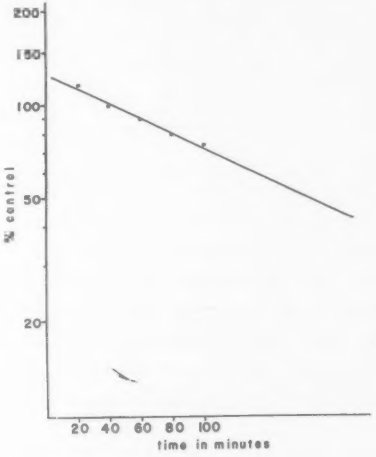


**Fig. 7.** Effect of thio-TEPA upon human leukemic leukocytes to produce logarithmic decay of oxygen uptake.

dependent curve. Evaluation of these effects would necessitate comparisons of the time to produce 50% inhibition as a function of chemical concentration, with extrapolation to a hypothetical zero time for 50% inhibition.

Table 2 is a summary of an experiment in which cells in homologous plasma obtained from a patient with subacute lymphatic leukemia were tested against a spectrum of chemicals. Note that the cells without added glucose respired at a rate less than 10% greater than those with added glucose. This is about as large a Crabtree effect as we have found in human leukemic leukocytes in homologous plasma. The time of contact with added chemicals and glucose was 90 min. All the rates were linear with time over this interval, with the exception of the cells which were incubated with nitrogen mustard. The rate for the nitrogen mustard–treated cells was linear up to 50 min. (58% of control; see Table 2) and then the respiration rate rapidly decreased.

In this experiment, as well as all others in which comparisons could be made, human leukemic cells were found to be most sensitive to the alkaloid, vinblastine sulfate; the alkylating agents triethylenethio-

**Table 2.** SENSITIVITY OF HUMAN LEUKEMIC LEUKOCYTES TO CHEMICALS

| *Conditions* | *Chemical conc. (μg./10⁶)* | *Respiration (μl. O₂/hr.*)* | *% Controls* |
|---|---|---|---|
| $230 \times 10^6$ cells | | 49 | |
| Cells + glucose | | 45 | |
| Cells + glucose + diazouracil | 22 | 45 | 100 |
| Cells + glucose + 8-azaguanine | 21.6 | 42 | 93 |
| Cells + glucose + 6-mercaptopurine | 21.9 | 44 | 98 |
| Cells + glucose + vinblastine sulfate | 3.3 | 34 | 75 |
| Cells + glucose + Meticorten | 20.4 | 42 | 93 |
| Cells + glucose + nitrogen mustard | 21.8 | 26 | 58 |
| Cells + glucose + triethylenethiophosphoramide | 11.9 | 34 | 75 |

*Calculated from 90-min. experiment where rates were linear with time for the 90-min. period, except for nitrogen mustard which gave linear rate for 50 min., then became nonlinear.

phosphoramide and nitrogen mustard rank next in order of decreasing effect. These results are in line with the observed clinical picture of severe bone marrow depression reported to occur from use of these compounds, especially vinblastine sulfate. Thus, the toxicity of these materials may be due to the fact that these materials destroy the energy generating mechanisms of normal cells as well as the tumor cells.

### The Effect of Chemicals upon Aerobic Glycolysis

It is well established that cancer cells convert glucose to lactic acid under aerobic conditions. The magnitude of lactic acid production may be seen in Table 3. Only the white blood cells from a patient with a leukemoid reaction due to reticulum cell sarcoma gave rates far in excess of those found for the leukemic cells. It is of interest to note that the possibility exists that normal leukocytes may have the capacity to convert glucose to lactic acid under aerobic conditions.

The effect of chemicals on the aerobic glycolysis of human leukemic leukocytes in homologous plasma is shown in Table 4, which is a representation of a typical experiment, and in Fig. 8, which is a summary of all the data.

The data in Column 4 of Table 4 are illustrative of the types of effects which we have noted to one degree or another with each chemical depending upon the chemical concentration.

It is noteworthy that nearly all the chemicals stimulate aerobic glycolysis. The exception is aminopterin, which we have found to have little or no effect over the concentration range studied (Fig. 8). Nitro-

**Table 3.** AEROBIC LACTIC ACID PRODUCTION

| *Condition* | *Lactic acid (μg./hr./10⁶ cells)* |
|---|---|
| Leukemoid reaction* | 230 |
| Stem cell leukemia* | 75 |
| Chronic lymphatic leukemia | 63 |
| Chronic lymphatic leukemia* | 70 |
| Subacute lymphatic leukemia† | 60 |
| Chronic lymphatic leukemia | 105 |
| Chronic lymphatic leukemia | 36 |

*Average of 2 experiments.
†Average of 4 experiments, including one with washed cells.

gen mustard will inhibit aerobic glycolysis, in general, though we have found instances where it was without effect or even produced some stimulation.

A most important feature is the lack of correlation between effect of a chemical upon respiration (Table 4, Column 5) and effect upon aerobic glycolysis. In contrast to aminopterin, which shows no effect, 6-mercaptopurine at a concentration which shows no effect upon respiration produces a relatively large stimulation of lactic acid production. At the other extreme, nitrogen mustard inhibits both respiration and lactic acid production, while triethylenethiophosphoramide stimulates lactic acid production at concentrations at which it inhibits respiration.

The effect of chemical concentration may best be summarized from

**Table 4.** EFFECT OF CHEMICALS ON AEROBIC GLYCOLYSIS OF HUMAN SUBACUTE LYMPHATIC LEUKEMIC CELLS IN HOMOLOGOUS PLASMA

| *Condition** | *Chemical concentration (μg./10⁶)* | *Lactic acid* | | *% respiration* |
|---|---|---|---|---|
| | | *Net synthesis (mg.)* | *Net change over control (mg.)* | |
| Control | | 0.44 | | |
| Aminopterin | 20 | 0.40 | −0.04 | 100 |
| Diazouracil | 23.5 | 1.33 | +0.89 | 67 |
| 8-Azaguanine | 23.2 | 1.00 | +0.56 | 95 |
| 6-Mercaptopurine | 22.5 | 0.94 | +0.50 | 99 |
| Nitrogen mustard | 22.8 | 0.24 | −0.20 | 29 |
| Triethylenethiophosphoramide | 11.3 | 1.41 | +0.97 | 40 |
| Vinblastine sulfate | 3.6 | 1.00 | +0.56 | 86 |
| Meticorten | 20.4 | 0.73 | +0.29 | 100 |

*222 $\times$ $10^6$ cells per Warburg flask incubated for 190 min. with glucose; chemical and chemical concentration as μg./$10^6$ cells are as noted.

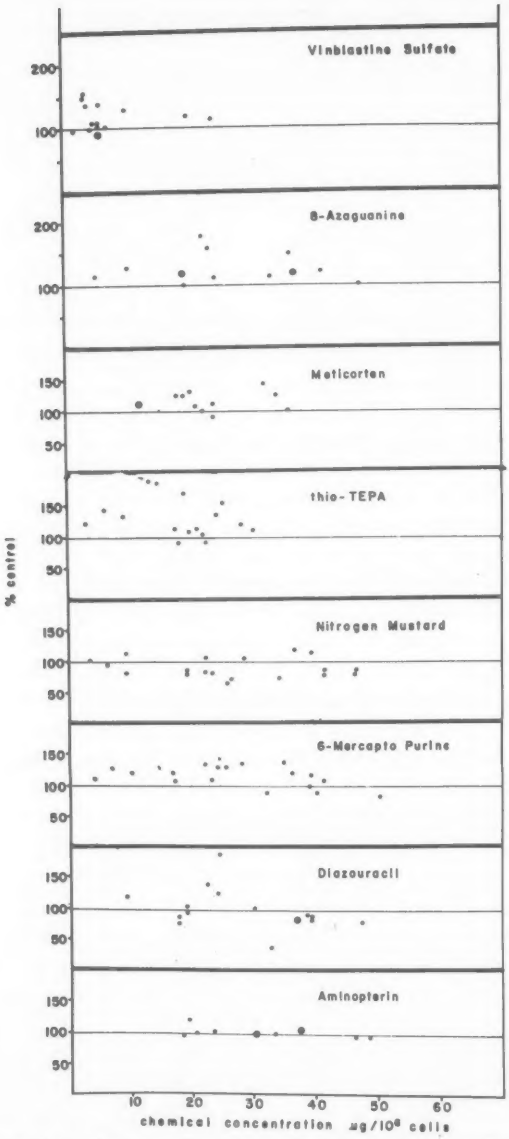


**Fig. 8.** Effects of chemicals upon aerobic glycolysis (lactic acid production) of human leukemic leukocytes in homologous plasma. Abscissa shows chemical concentration as $\mu g./10^6$ cells; ordinate for each chemical ranges from zero to 200% of control.

the data given in Fig. 8, where we have plotted percentage of control of lactic acid versus chemical concentration for all the data.

In general, at concentrations greater than 40 $\mu$g./$10^6$ cells, all the chemicals studied will probably inhibit aerobic glycolysis. It is noteworthy that at the other end of the spectrum, even at concentrations less than 10 $\mu$g./$10^6$ cells, many of the chemicals stimulate lactic acid production. The chemicals which produce stimulation do so over a wide range of concentrations, and there is some tendency to a particular peak value for each of the chemicals.

Aminopterin appears to be without effect over the range studied; nitrogen mustard shows inhibition over this range. The scatter of points above and below 100% exhibited by diazouracil cannot be explained.

Since the data obtained for aerobic lactic acid production represent the total change effected over the experimental time period, the kinetics of the reaction cannot be evaluated. However, it is apparent that stimulation of lactic acid production occurs at chemical concentrations which do not have any effect on respiration. But when inhibition occurs, as with the nitrogen mustard, it occurs only when there is inhibition of respiration.

### Effect of Chemcals upon Total Carbohydrate Potential of Cancer Cells

A balance sheet was kept of the dissimilation of glucose in the presence and absence of chemicals. In all instances, with the exception of 6-mercaptopurine-treated cells, the glucose which was used up during the experiment could be accounted for in terms of oxygen uptake and lactic acid produced. In all the experiments in which 6-mercaptopurine was used there was a greater disappearance of glucose than could be accounted for as in the other instances. This effect showed up in some experiments when triethylenethiophosphoramide was used. The effect of 6-mercaptopurine was also found to occur when cells from a transplantable chloromyeloid leukemia carried in Sprague-Dawley rats were tested; these results will be reported elsewhere. The effect of 6-mercaptopurine is so unusual that we will discuss it alone.

A typical balance sheet is shown in Table 5, the last column of which records the difference found between glucose utilized and glucose dissimilated (see table for definitions). It can be seen that 2.1 mg. of glucose is unaccounted for in the presence of 6-mercaptopurine and 0.50 mg. is unaccounted for in the presence of triethylenethiophosphoramide.

**Table 5.** Balance of Glucose Utilization in Human Leukemic Leukocytes in Homologous Plasma

| *Condition* | *Chemical concentration* ($\mu g./10^6$ *cells*) | *Glucose* | | *Differences* (*mg.*) |
|---|---|---|---|---|
| | | *Utilized** (*mg.*) | *Dissimilated†* (*mg.*) | |
| Control | | 0.20 | 0.22 | 0.02 |
| Diazouracil | 22 | 0.57 | 0.51 | 0.06 |
| 8-Azaguanine | 21.6 | 0.66 | 0.75 | 0.09 |
| 6-Mercaptopurine | 21.9 | **2.56** | 0.46 | **2.10** |
| Nitrogen mustard | 21.8 | 0.20 | 0.16 | 0.04 |
| Triethylenethiophosphoramide | 10.9 | **1.21** | 0.71 | **0.50** |
| Vinblastine sulfate | 3.3 | 0.60 | 0.59 | 0.01 |

*Glucose in flask at zero time minus glucose in flask at end of experiment.

†Sum of glucose equivalents used in respiration and glucose equivalents of lactic acid produced.

This effect of 6-mercaptopurine was found in every experiment carried out, regardless of the cell type, chemical concentration, or time of incubation. The effect with triethylenethiophosphoramide was found only in those experiments where the incubation time exceeded 2 hr.

## Discussion

The respiratory activity of human leukocytes, within the limitations of our investigation, may be divided into two categories. The separation into two categories of high-activity group and low-activity group is easily achieved because the differences between groups is quite marked, the activity of the former being 2–3 times the latter. The blast cell shows high activity regardless of site of origin. The other cell types designated as chronic lymphatic or chronic myelogenous, or leukemoid reaction show low respiratory activities. The ability to distinguish between cell types in terms of respiratory activity may serve as an adjunct to established diagnostic procedures. It is notable that in one case diagnosed as subacute lymphatic leukemia by our criteria, the respiratory activity would predict chronic lymphatic leukemia. From the clinical standpoint, the course of therapy would be less ambiguous had it been possible to make a diagnosis of "acute" or "chronic." The practical value of the respiratory activity in clinical diagnosis, however, will have to wait until a larger body of relevant information is available than is now.

The clinical usefulness of a cell sensitivity test is obvious. At the moment, however, a sensitivity test has not been developed that pro-

vides the absolute quantitative data one would like to have, in the sense that one could calculate the exact dosage to be administered to a patient. Nevertheless, certain qualitative relationships are readily discernible which are of significance and should contribute useful information to the physician in planning a course of chemotherapy. For example, from Fig. 3, it can be seen that the relative sensitivity (concentration to produce 50% inhibition) for triethylenethiophosphoramide, 8-azaguanine, and aminopterin is 10, 4, and 1, respectively. Such relationships are readily obtained for any series of chemotherapeutic agents adjudged to be drugs of choice by the physician. As a result of such a test, dosages or therapeutic regimen could be adjusted to provide the maximum effects with a minimum toxicity based upon relative sensitivity indices; whether a 50% or 75% index is used remains at the moment in the realm of clinical judgment.

One obvious question which the cell sensitivity study brings into focus is what is a "good" chemotherapeutic agent. If the criterion is ability to "kill" tumor cells, then in general the alkylating agents are "good." However, if "kill" occurs, as was demonstrated, by destruction of aerobic energy-generating mechanisms, then the alkylating agents have to be considered as toxic to normal tissue and, therefore, not "good." In this same sense, the antimetabolites are "good." From a clinical standpoint, knowledge of the relative toxicity of these drugs with respect to a particular patient might permit a wider latitude in the choice and use of agents.

If the cell respiratory sensitivity index, as we suspect, ultimately reflects toxicity, then it seems reasonable that this simple index should be determined directly on human tumor tissue and perhaps used in the final evaluation of new chemicals in the chemotherapeutic screening program.

The stimulation of aerobic lactic acid production by nearly all the chemicals tested is of particular interest with respect to cell metabolism.

Both the Pasteur effect (inhibition of glucose utilization by oxygen) and the Crabtree effect (inhibition of respiration by glucose) have been reviewed recently (*10*). If, as reported by Quastel and Bickis (*11*), the energy lost by glucose-suppressed respiration is exactly replaced by glycolytically formed high-energy phosphate, then an increase in lactic acid production caused by a chemical should be accompanied by further inhibition of oxygen uptake. However, such results were not always obtained. Further, if tight coupling between respira-

tion and glycolysis obtains for the cell in the absence of added chemical, then one may construe that the effect of the chemical cannot be to produce "uncoupling," as this would cause stimulation of respiration and reduction of lactic acid production.

The explanation of our observed chemical effects upon respiration and aerobic glycolysis is more plausible when viewed from the standpoint of alteration in the Pasteur effect.

We have observed that stimulation of aerobic lactic acid produced by chemicals may or may not accompany inhibition of respiration. Either situation is compatible with a mechanism in which the lactic dehydrogenase is loosely coupled to respiration, so that (1) with concomitant inhibition of respiration more pyruvate is available to be shuttled through to lactate, and (2) without concomitant inhibition the enzyme lactic dehydrogenase may be stimulated by the chemical agent, though under aerobic conditions, to the maximum anaerobic rate of enzymatic activity. The effects discussed above are to a certain degree concentration-dependent; for example, triethylenethiophosphoramide at low concentrations stimulates lactic acid production without inhibition of respiration, but at higher concentrations does inhibit respiration. True Pasteur effect inhibitors stimulate lactic acid production without inhibition of respiration (*12*). However, in view of the concentration effect noted above, this definition might be construed as being too narrow. Nevertheless, we see that, with the possible exception of nitrogen mustard, all the other chemicals tested can be classified as Pasteur effect inhibitors.

It has been proposed by Boxer and Devlin (*13*), that the ultimate chemical for truly specific inhibition of tumor activity without concomitant effect upon normal cellular activity will be the chemical that inhibits irreversibly lactic dehydrogenase. If this in fact be true, then the chemotherapeutic agents now in use are operating in the opposite direction.

In addition to the stimulation of aerobic lactic acid, we have shown that 6-mercaptopurine has the unique property of stimulating glucose disappearance to products not yet identified.

Pecile and co-workers (*14*) have reported that 4 days' treatment of tumor-bearing mice with 100 mg./kg. intraperitoneal injections of 6-mercaptopurine brings about increased glycogen synthesis activity of Ehrlich ascites tumor cells. This is shown by a higher glycogen content of tumor cells and by the deposition of glycogen following intraperitoneal glucose administration on the fourth day of chemical treat-

ment. This result was interpreted in terms of the selective inhibition of tumor lactic dehydrogenase found after 6-mercaptopurine treatment for 10 days at a dose of 100, 50, and 25 mg./kg. (*15*).

While direct stimulation of glycogen synthesis cannot be ruled out as an explanation for glucose disappearance in human tumor cells, it is possible that under the conditions of our experiments, which were about one hour in duration, in vitro, and at concentrations where lactic acid production was stimulated and not inhibited, the increased glucose disappearance is brought about by an enhanced hexokinase reaction and conversion to glucose-6-phosphate as the primary site of action. The mechanism of action of 6-mercaptopurine is under investigation.

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# The Effect of Fatty Acids upon Tumor Cell Respiration and Transplantability

*Bernard J. Katchman, Robert E. Zipf, and James P. F. Murphy*

**The kinetic effect of palmitate, stearate, oleate, linoleate, and linolenate upon in vitro endogenous respiration of rat chloromyeloid leukemic cells has been investigated.**

**Inhibition of respiration has been correlated with the ability of fatty acids to cause decreased cell viability and cell count; in the bioassay of fatty acid–treated tumor inocula, the increase in animal life span is correlated to the degree of dilution of the inocula due to cell lysis.**

**The degree of lysis is dependent upon the chemical structure of the fatty acid, concentration, and duration of contact; unsaturated fatty acids are more effective than saturated fatty acids.**

**Tumor cells, when incubated at low concentrations of fatty acids, show stimulation of $O_2$ uptake; however, in the bioassay these fatty acid–treated inocula showed no loss in tumor activity.**

**The nature of the physiochemical interaction between fatty acids and tumor cells is discussed.**

As part of a study of the effects of chemicals upon human and animal tumor cells, we investigated the effects of several fatty acids upon the endogenous respiration and transplantability of rat leukemic cells (actinium-227–induced transplantable chloromyeloid leukemia carried in Sprague-Dawley rats) (*1*).

It has been reported (*2*) that the active principle of royal jelly which shows antitumor activity (preincubation of tumor inoculum) is the fatty acid, 10-hydroxy-2-decenoic acid, and it has been proposed that such materials be investigated for their clinical usefulness. Bennett

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and Connon (*3*) have shown that fatty acids have a potent lytic action upon cells; also, that the degree of lysis may be correlated to the degree of inhibition of respiration.

Morgan *et al.* (*4*), in a study of saturated dicarboxylic acids, concluded that the mechanism by which dicarboxylic acids inhibit tumor formation (preincubation) has not been elucidated.

In this report, we present the results of our studies with animal tumor cells exposed to a variety of fatty acids, over several decades of concentration, as a function of time. The effects upon endogenous respiration, cell count, viability, and transplantability have been recorded, analyzed, and correlated.

## Materials

*Krebs-Ringer phosphate buffer* (*5*), p*H 7.2*

*Physiological saline* 0.9% sodium chloride in water.

*Erythrosin-B* 0.4% solution in water (Harleco, Philadelphia).

*Nigrosin, certified, water soluble* 0.5% in Krebs-Ringer phosphate buffer, *p*H 7.2 (Allied Chemical and Dye Corp., New York).

*Fatty Acids* Fatty acids used were of the highest grade commercially available. Stearic, palmitic, oleic, linoleic, and linolenic acids were converted to sodium salts by addition of equivalent amounts of sodium hydroxide, dissolved in water, and then back-titrated with acid such that subsequent dilutions with buffer gave solutions with *p*H 7.2.

## Methods

### Preparation of Tumor Cell Suspension

The tumor-bearing rat is ether anesthetized and exsanguinated by heart puncture. The tumor mass is collected in a petri dish and washed with 10 ml. of physiologic saline, and the necrotic tissue is removed. The tumor mass is minced with scissors in a petri dish to release cells. By using a stainless-steel wire gauze (40 mesh per inch) with the same inside diameter as the petri dish, the released cells can be separated from the tumor mince and collected in a syringe fitted with a 20-gauge needle. This operation is carried out 3 times with 10-ml. portions of physiologic saline.

The cell suspension is collected in a 15-ml., plastic test tube and centrifuged at 1000 rpm for 10 min. at room temperature. The supernatant is discarded and the upper erythrocyte layer removed. The cell mass is resuspended in an equal volume of water for 30–60 sec. (to cause lysis of the remaining erythrocytes), and then diluted with 10

volumes of physiologic saline, centrifuged, washed, and resuspended; this is repeated 3 times, and then it is diluted to a 25- to 50-ml. volume.

Cell counts are performed routinely using standard hemocytometer technics. Aliquots of the washed cell suspension ($100 \times 10^6$ cells) are taken for study.

Viability was determined by two stain methods: (1) erythrosin-B stain by the method of Phillips and Terryberry (*6*) and (2) nigrosin stain by the method of Kaltenbach *et al.* (*7*). In the former, two parts of cell suspension and one part of 0.4% stain solution were found to give best results. Counting of the stained and unstained cells was done in an improved Neubauer ruled standard blood-counting chamber. With the second method, cells were diluted twentyfold in a white-cell–diluting pipet using nigrosin solution (0.5% nigrosin in Krebs-Ringer phosphate buffer) as the diluent. Cell counts were made in the improved Neubauer ruled blood-counting chamber.

### Warburg Manometric Technique

Respiratory $O_2$ uptake was determined by the method as outlined in Umbreit (*8*). For the measurement of $O_2$ uptake, the following additions were made to the Warburg flasks: in the center well, 0.2 ml. of 20% potassium hydroxide; in the main flask, aliquots of cell suspension of about $100 \times 10^6$ cells in Krebs-Ringer phosphate buffer, *p*H 7.2. An aliquot of buffered fatty acid solution was placed in the side arm of the flask and tipped in at zero time; total flask volume was made up to 3.2 ml. with buffer solution.

Flasks were attached to manometers and equilibrated in the water bath at 37.5° for 10 min. before manometric readings were begun. Manometric experiments were carried out for 1–2 hr.

### Bioassay

Cell suspensions of about $100 \times 10^6$ cells were incubated at room temperature with buffered solutions of selected fatty acids at concentrations for various time intervals. At the end of the incubation period, the cell suspensions were centrifuged, the fatty acid containing supernatant discarded, and the cells resuspended in buffer solution. Aliquots were then diluted in buffer solution to about $5 \times 10^6$ cells per milliliter and 0.2 ml. injected into 10- to 12-day-old Sprague-Dawley rats. Both control and fatty acid–treated cell suspensions were counted and stained for viability at each experimental time period.

## Results

### In Vitro Metabolic Studies

The metabolic activity of rat chloromyeloid tumor cell suspensions is summarized as follows.

$Q_{O_2}$ = 3.4 $\mu$l. $O_2$ consumed/mg. dry weight of cells/hr.

$Q_{LA}$ = 18.3 $\mu$l. $CO_2$ calculated from lactic acid production/mg. dry weight of cells/hr.

$Q_{LA}^{N_2}$ = 27.3 $\mu$l. $CO_2$ directly observed or calculated from lactic acid production/mg. dry weight

(Aerobic metabolic studies in Krebs-Ringer phosphate with air in gas phase and 0.2 ml. 20% KOH in center well; 0.2 ml. of 0.1M glucose. Total volume 3.2 ml. Anaerobic experiments in Krebs-Ringer bicarbonate with 95% $N_2$ — 5% $CO_2$ in gas phase; 0.2 ml. of 0.1M glucose. Total volume 3.2 ml.)

The pattern of activity of these tumor cells fits that reported for other animal tumor systems. In addition to the high aerobic glycolysis, as seen above, these cells also show the Crabtree effect (*9*) (inhibition of respiration by glucose).

Low-molecular-weight organic acids have no measurable effects upon endogenous respiration at the concentrations studied. These include succinate, $\alpha$-ketoglutaric, citrate, oxalacetate, malate, aconitate, acetate, $\beta$-hydroxyproprionate, and acetoacetate.

However, fatty acids such as stearate, palmitate, oleate, linoleate, and linolenate do affect respiration to varying degrees depending upon concentration (moles of fatty acid per $10^6$ cells), duration of contact with fatty acid, and the chemical structure of the fatty acid.

The effects of fatty acids upon tumor respiration are concentration- and time-dependent. Figure 1 is a representation of a typical experiment. The concentrations of fatty acid are normalized as moles of fatty acid per $10^6$ cells. The control rate of $O_2$ uptake is constant, which is indicative of zero-order reaction rate with respect to $O_2$ uptake. At the lowest fatty acid concentration ($1.28 \times 10^{-9}$ moles per $10^6$ cells), there is initially a slight stimulation of $O_2$ uptake followed by a nonlinear inhibitor effect which becomes apparent after 40 min. of contact with fatty acid. At higher concentrations, there develops a family of nonlinear inhibition curves which are concentration-dependent. Although these nonlinear curves are likely to be representative of a complex series of reactions, it can be shown that over most of the experimental time intervals, the curves are logarithmic decay curves. When remaining activity (%) is plotted against time on semilog paper (log

% activity versus time), a straight line is obtained. This is shown in Fig. 2. Note that the family of nonlinear curves of Fig. 1 is transformed to a family of linear curves. It is obvious that the measured effects are complex. If the reactions were simply logarithmic, the lines should

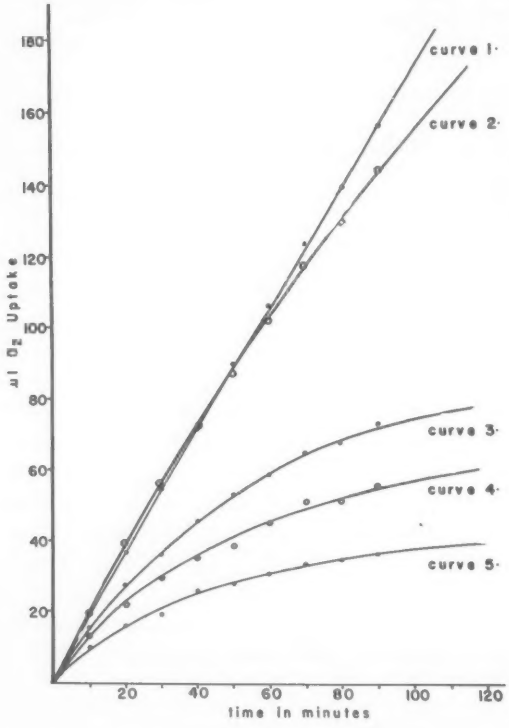


**Fig. 1.** Effect of oleic acid upon endogenous respiration of tumor cell suspension. Curve *1*, control; Curve *2*, $1.28 \times 10^{-9}$ moles/$10^6$ cells; Curve *3*, $2.56 \times 10^{-9}$ moles/$10^6$ cells; Curve *4*, $3.84 \times 10^{-9}$ moles/$10^6$ cells; Curve *5*, $5.12 \times 10^{-9}$ moles/$10^6$ cells.

intersect 100% at zero time. In a sense, this is true only for the lowest concentration (Curve I). In Fig. 1, it can be seen that only after 40 min. of contact with the oleic acid, did the inhibition of $O_2$ uptake become measurable. At this concentration in Fig. 2, the curve extrapolates back to 100% activity remaining at about 40 min. At the higher concentrations, the curves of Fig. 2 do not extrapolate to 100%, and actually each curve does extrapolate to a lower point on the ordinate as the concentration increases. The initial effects at high concentrations are such as to produce an apparent rate of inhibition greater than that

found after 20 min. when the straight-line patterns of the curves are demonstrable.

From these curves (Fig. 2), it is possible to calculate the time for 50% inhibition, $t_{1/2}$, to take place. These data for five long-chain fatty

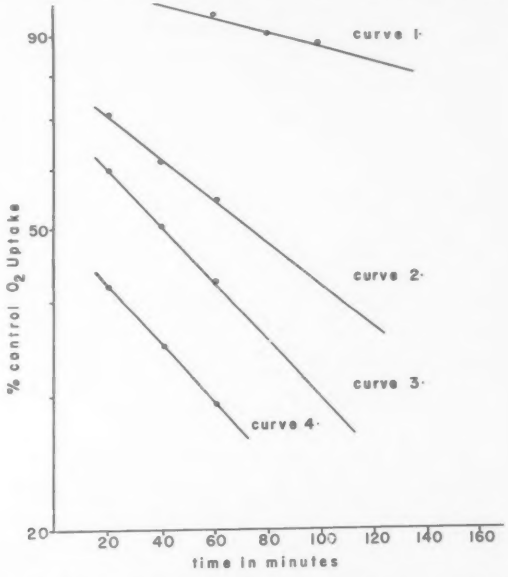


**Fig. 2.** Plot of log % control activity versus time for data in Fig. 1. Curve *1*, 1.28 $\times$ $10^{-9}$ moles/$10^6$ cells; Curve *2*, 2.56 $\times$ $10^{-9}$ moles/$10^6$ cells; Curve *3*, 3.84 $\times$ $10^{-9}$ moles/$10^6$ cells; Curve *4*, 5.12 $\times$ $10^{-9}$ moles/$10^6$ cells.

acids are summarized in Table 1. The relationships which exist between $t_{1/2}$ for a particular fatty acid as a function of concentration and the different fatty acids are readily discerned in Fig. 3, where $t_{1/2}$ is plotted against concentration over three decades. First, it can be seen that for each fatty acid, $t_{1/2}$ is linear with concentration. This means that under the conditions of the experiment, a relative inhibition index can be estimated. If one extrapolates the straight lines to $t_{1/2}$ equal to zero, one will obtain the concentration which will theoretically produce instantaneous inhibition of 50% the activity. In Table 2, it can be seen that palmitic acid, a $C_{16}$ saturated acid, is more effective than stearic acid, a $C_{18}$ saturated acid. Again, comparison of the four $C_{18}$ fatty acids shows that, as the unsaturation increases (0, 1, 2, 3 double bonds in the molecule), the effectiveness to produce inhibition increases. However, linolenic (3 double bonds) acid is only slightly more effective than linoleic acid.

**Table 1.** INHIBITION OF RESPIRATION BY FATTY ACIDS

| *Fatty acid* | *Conc. (moles F. A./$10^6$ cells)* | *$t_{1/2}$ (min.)* |
|---|---|---|
| Stearic acid ($C_{18}H_{36}O_2$) | $4.5 \times 10^{-8}$ | 145 |
| | $8.9 \times 10^{-8}$ | 95 |
| | $13.3 \times 10^{-8}$ | 57 |
| Palmitic acid ($C_{16}H_{32}O_2$) | $2.88 \times 10^{-8}$ | 116 |
| | $4.1 \times 10^{-8}$ | 68 |
| | $5.76 \times 10^{-8}$ | 70 |
| | $5.4 \times 10^{-8}$ | 64 |
| | $8.0 \times 10^{-8}$ | 47 |
| | $8.3 \times 10^{-8}$ | 35 |
| | $12.4 \times 10^{-8}$ | 15 |
| Oleic acid ($C_{18}H_{34}O_2$) | $0.39 \times 10^{-8}$ | 234 |
| | $0.78 \times 10^{-8}$ | 85 |
| | $1.14 \times 10^{-8}$ | 52 |
| | $1.53 \times 10^{-8}$ | 12 |
| Linoleic acid ($C_{18}H_{32}O_2$) | $0.15 \times 10^{-8}$ | (800) |
| | $0.46 \times 10^{-8}$ | 70 |
| | $0.92 \times 10^{-8}$ | 20 |
| Linolenic acid ($C_{18}H_{30}O_2$) | $0.52 \times 10^{-8}$ | 64 |
| | $0.87 \times 10^{-8}$ | 7 |

For the fatty acids at concentrations lower than that required for inhibition, by a factor of approximately 10, one can demonstrate stimulation of $O_2$ uptake. Stimulation of $O_2$ uptake is concentration- and time-dependent. In Table 3, are summarized our data which demonstrate this effect. The stimulating effect is of the order of 10–20%. It is concentration-dependent (see palmitate data), and it is also dependent upon the type of chemical: compare stearic acid and linoleic acid. It is also time-dependent. This means that the stimulation effect gradually grades into inhibition. The concentration of fatty acid, of course, determines just how long it takes for inhibition to begin to operate. This is shown in Fig. 1 (oleic acid) and Table 3 for the data with linoleic acid at $0.15 \times 10^{-8}$ moles fatty acid per $10^6$ cells. The value (800 min.) is the value for $t_{1/2}$ estimated from an inhibition curve.

The stimulation of $O_2$ uptake by fatty acids has been interpreted in terms of an uncoupling effect of oxidative phosphorylation in the mitochondria (*10*). This uncoupling effect was found to be reversible in isolated mitochondria (*11*). However, it can readily be shown that with intact cells the stimulation effect has a finite time and transitory existence, and then begins to grade into inhibition.

The effect of fatty acids, fatty acid concentration, and length of con-

tact of tumor cells upon cell count and viability is shown in Table 4. In evaluating Table 4, it should be noted that, although at the end of the experimental time period the cells were separated from the fatty acid supernatant in which they were suspended, there is no way of assessing the amount of fatty acid remaining on the cell surface. Since there is a finite time required for cell counts and viability studies to be effected, one cannot exactly quantitatively correlate these microscopic studies with respiration effects.

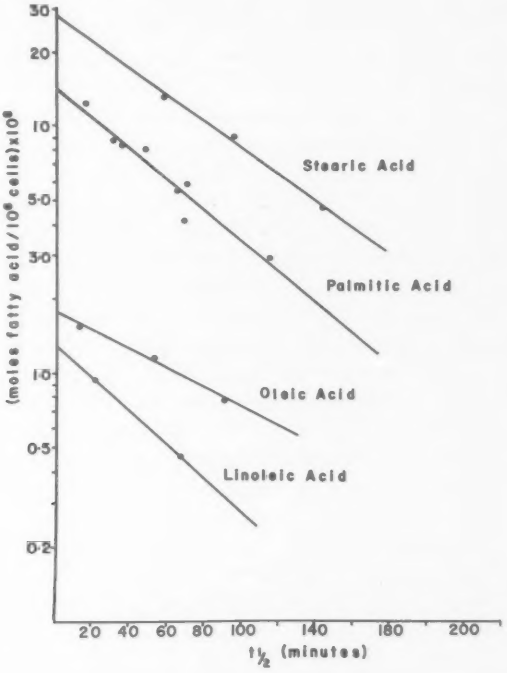


**Fig. 3.** Time for 50% inhibition ($t_{1/2}$) versus concentration of fatty acid.

**Table 2.** Relative Inhibition Index of Fatty Acids

| *Fatty acid* | *Double bond* | *Conc. (moles F. A./10⁶ cells at $t_{1/2} = 0$)* |
|---|---|---|
| Palmitic acid ($C_{16}H_{32}O_2$) | 0 | $14 \times 10^{-8}$ |
| Stearic acid ($C_{18}H_{36}O_2$) | 0 | $28 \times 10^{-8}$ |
| Oleic acid ($C_{18}H_{34}O_2$) | 1 | $1.75 \times 10^{-8}$ |
| Linoleic acid ($C_{18}H_{32}O_2$) | 2 | $1.25 \times 10^{-8}$ |
| Linolenic acid ($C_{18}H_{30}O_2$) | 3 | $0.91 \times 10^{-8}$ |

**Table 3.** STIMULATION OF OXYGEN UPTAKE BY FATTY ACIDS

| *Acid* | *Control* (*μl. $O_2$/hr./$10^6$ cells*) | *Experimental* (*μl. $O_2$/hr./$10^6$ cells*) | *Concentration moles F. A./$10^6$ cells* |
|---|---|---|---|
| Stearic | 1.0 | 1.19 | $1.5 \times 10^{-8}$ |
| Palmitic | 0.70 | 0.76 | $0.37 \times 10^{-8}$ |
| | | 0.92 | $0.28 \times 10^{-8}$ |
| | | 0.75 | $0.14 \times 10^{-8}$ |
| | | 0.72 | $0.05 \times 10^{-8}$ |
| | 0.77 | 0.85 | $0.48 \times 10^{-8}$ |
| | 0.83 | 0.90 | $0.95 \times 10^{-8}$ |
| Linoleic | 0.81 | 0.93 | $0.3 \times 10^{-9}$ |
| | 0.88 | 1.01 | $0.3 \times 10^{-9}$ |
| | 0.89 | 0.99 | $0.31 \times 10^{-9}$ |

The first effects produced by the fatty acids are upon viability. The viability decreases before cell count decreases as would be expected if the initial effect of the fatty acid was on cell permeability. The effect of time upon viability at any concentration is readily seen as is the effect of concentration of fatty acid at any particular time. Within certain limits, one can associate these effects upon viability and cell count with the measured respiratory activity. At the lowest concentration, the cell count and viability are intact at the end of 30 min. (stimulation of respiration); at the end of 60 min., only a slight effect upon viability

**Table 4.** EFFECT OF FATTY ACIDS UPON VIABILITY AND CELL COUNT

| | *Concentration (moles F. A./$10^6$ cells)* | | | | |
|---|---|---|---|---|---|
| | *Control* | *$1.44 \times 10^{-8}$* | *$2.88 \times 10^{-8}$* | *$5.6 \times 10^{-8}$* | *$8.64 \times 10^{-8}$* |
| | | | 0 TIME | | |
| Cell count $\times 10^6$ | 35 | | | | |
| Viability (%) | 98 | | | | |
| | | | 30 MIN. | | |
| Cell count $\times 10^6$ | 34 | 36 | 25 | 16 | Clumped, viscous |
| Viability (%) | 83 | 89 | 91 | 36 | |
| | | | 60 MIN. | | |
| Cell count $\times 10^6$ | 32 | 34 | 28 | Clumped, viscous | Clumped, viscous |
| Viability (%) | 96 | 82 | 40 | | |
| | | | 90 MIN. | | |
| Cell count $\times 10^6$ | 28 | 27 | 18 | | |
| Viability (%) | 90 | 35 | 12 | | |

with still no measurable effect upon respiration; at the end of 90 min., the effect upon cell count and viability is prominent, and this is reflected by logarithmic decay of respiratory activity. At concentrations lower than those shown in Table 4, sustained $O_2$ stimulation for 120 min. was achieved without any effect upon cell count or viability.

The relative effect of fatty acid upon cell wall permeability may be demonstrated with Table 5. Note that control cells show equal permeability or lack thereof to both erythrosin-B and nigrosin stain. However, the cells treated with fatty acids show greater uptake of erythrosin-B than nigrosin. While this type of data casts certain doubts as to the validity of the staining technique for viability, in any absolute sense, it does show or indicate the possible role that fatty acids may play in loosening of the cell membrane as a prelude to generalized lysis.

### Effect of Fatty Acid Incubation on Tumor Cell Transplantability

The effect of preincubation of tumor cell inocula with fatty acids upon the life span of injected animals was also investigated. It can be demonstrated by means of Table 6 that, under selected conditions of concentration of fatty acids and incubation time with tumor cells, the increase in mean life span of injected animals is readily correlated to the degree of cell lysis. It has already been shown that inhibition of respiration may be correlated to loss of cell viability and eventually to a loss in cell count due to lysis. The data in Table 6 are also in agreement with the results reported previously (*1*) of the effect of dilution of tumor inocula upon mean life span: dilution of tumor inocula produces an increase in mean life span as well as a decrease in the percentage of "tumor takes."

Some of the data in Table 6 need further clarification. First, at the two higher concentrations, except where the notation "clumping" appears, there were no cells observed microscopically in the incubated samples, and therefore, no injections were made. Second, depending

**Table 5.** Comparison of Erythrosin-B- and Nigrosin-Stained Cells

| | *Viability (%)* | |
|---|---|---|
| | *Erythrosin blue* | *Nigrosin* |
| Control, 0 time | 92 | 95 |
| Treated, 30 min. | 77 | 92 |
| Treated, 90 min. | 32 | 87 |

Linoleic acid at $0.16 \times 10^{-9}$ moles/$10^6$ cells.

**Table 6.** Effect of Linoleic Acid Incubation on Tumor Cell Transplantability

| | *Concentration (moles F. A./$10^6$ cells)* | | | |
|---|---|---|---|---|
| | *Control* | *0.294 × $10^{-7}$* | *0.147 × $10^{-6}$** | *0.294 × $10^{-6}$** |
| | | 0 Time | | |
| Cell count† × $10^6$ | 23.8 | 19.4 | Clumping | No cells |
| Mean life span, days | 35 | 70 | | |
| Animals dead/animals injected | *8*<br>8 | *7*<br>9 | | |
| | | 1 Hr. | | |
| Cell count × $10^6$ | 27.4 | 7.7 | No cells | No cells |
| Mean life span, days | 40 | 137 | | |
| Animals dead/animals injected | *10*<br>10 | *3*<br>10 | | |
| | | 2 Hr. | | |
| Cell count × $10^6$ | 25.6 | 1.8 | No cells | No cells |
| Mean life span, days | 42 | | | |
| Animals dead/animals injected | *8*<br>8 | *0*<br>10 | | |

*Solutions not used for injections.

†For animal injections, aliquots of cell suspension taken such that control injection contained 1 × $10^6$ cells, and the same volume was used for experimental injections.

upon fatty acid concentration and incubation time, the cell suspensions developed, to varying degrees, unusually high viscosity. The lytic action which produces this viscous effect is so rapid and so complete that we routinely use fatty acids to lyse tumor cell suspensions for desoxyribonucleic acid extractions (*12*).

The lytic effect of relatively high concentrations of fatty acids upon tumor cell inocula was mainly to reduce the effective cell concentration as though a dilution had been effected. From the data of Bennett and Connon (*13*), it can be deduced that these long-chain fatty acids at the relatively high concentrations mentioned above would also be lytic for red cells as well as the tumor cells; therefore, the protective effect of the oleic acid with respect to its potential usefulness as a chemotherapeutic drug becomes meaningless. It was thought that, at concentrations of fatty acids which do not cause lysis or hemolysis but do affect respiration (stimulation of $O_2$ uptake as the causative factor in uncoupling of phosphorylation in the mitochondria), a selective type of inhibition of tumor cells might be effected.

**Table 7.** Effect of Incubation of Fatty Acid Upon Tumor Cell Respiration, Lactic Acid Production,* and Transplantability

| | *Incubation time* | | |
|---|---|---|---|
| | *0 Time* | *45 min.* | *90 min.* |
| Linolenic acid ($0.33 \times 10^{-9}$ Moles/$10^6$ Cells) | | | |
| Mean life span, days | | | |
| Control | 39 | 40 | 39 |
| Treated | | 39 | 39 |
| Animals dead/animals injected | | | |
| Control | *15* | *13* | *14* |
| | 16 | 14 | 15 |
| Treated | | *15* | *13* |
| | | 15 | 14 |
| Respiration, % control | | 100 | 100 |
| Lactic acid production, % control | | | 103 |
| Stearic acid ($0.85 \times 10^{-8}$ Moles/$10^6$ Cells) | | | |
| Mean life span, days | | | |
| Control | 33 | 33 | 31 |
| Treated | 33 | 33 | 34 |
| Animals dead/animals injected | | | |
| Control | *7* | *16* | *10* |
| | 7 | 16 | 10 |
| Treated | *11* | *13* | *11* |
| | 11 | 14 | 11 |
| Respiration, % control | | 100 | 100 |
| Lactic acid production, % control | | | 100 |
| Palmitic acid ($0.89 \times 10^{-8}$ Moles/$10^6$ Cells) | | | |
| Mean life span, days | | | |
| Control | 32 | 33 | 33 |
| Treated | 33 | 35 | 33 |
| Animals dead/animals injected | | | |
| Control | *21* | *13* | *10* |
| | 21 | 13 | 10 |
| Treated | *12* | *13* | *15* |
| | 12 | 13 | 15 |
| Respiration, % control | | 110 | 110 |
| Lactic acid production, % control | | | 135 |

*Lactic acid experiments in presence of added glucose; assayed by Barker and Summerson method (*14*); duration of experiments was 68, 140, and 108 min. for linolenic, stearic, and palmitic acids, respectively.

It can be seen in Table 7 that there is no effect upon the mean life span for linolenic acid, stearic acid, or palmitic acid. It is apparent that the uncoupling of phosphorylation produced by the palmitic acid is reversible in the intact cell as in the mitochondria (*11*). Thus even after 90 min. incubation when the palmitate-treated tumor inoculum is injected into animals, there must be physical or metabolic dilution of the fatty acid such that the mitochondria recover their capacity to carry out normal oxidative phosphorylation. This is, of course, reflected in the ability of the treated inoculum to produce the same degree of transplantability as the control inoculum. It is also notable that the palmitate incubation also produces stimulation of aerobic lactic acid production (135% of control), and that this "uncoupling" of glycolysis is either without effect upon tumor cell transplantability or, again, is indicative of a reversible effect.

## Discussion

The effect of long-chain fatty acids upon respiratory activity of tumor cells in vitro is shown to be concentration- and time-dependent. In any attempt to assess concentration effects, and this is obvious from our data, it is necessary to make comparisons at known equilibrium ratios of fatty acids to cells and to any other fatty acid binding materials that may be present; for example, red cells or blood proteins. It is easy to show that, at a molar concentration of fatty acid that causes lysis at a particular tumor cell count, increasing the tumor cell concentration will alter quantitatively, if not entirely qualitatively, the extent of the lytic effect.

Thus, it is reasonable to predict that in a solution of fatty acid in which tumor cells are suspended, an equilibrium will be established such that there will be a definite partition of the fatty acid (partition coefficient) between tumor cell (solid phase) and solution (buffer). The relative power of the different fatty acids to produce respiratory inhibition or lysis may therefore reflect differences in the partition coefficient for each of the fatty acids.

It is apparent, also, that even at relatively low concentrations of fatty acids, more than surface absorption occurs. The uncoupling of respiration is presumed to occur in the mitochondrial wall where the respiratory enzymes are found. Thus, there is a degree of cell permeation by the fatty acid. When the conditions are such that irreversible damage to the integrity of the cell has not been caused, then appar-

ently the fatty acid may be, subsequently, physically or metabolically washed out (*11*), and normal cell replication may take place. This is evident from the bioassay studies.

At the higher concentrations of fatty acids, cell damage, as seen from decreased cell count and stain viability, is irreversible; this is also confirmed by bioassay.

Our results are in qualitative agreement with the report of Bennett and Connon (*3*). However, quantitative comparison cannot be made because these authors used the water-insoluble fatty acids per se, solubilizing them with alcohol, whereas we used the soluble sodium salts.

The antitumor activity of fatty acids as found for 10-hydroxy-2-decenoic acid and other saturated dicarboxylic acids was reported (*2, 4*) to occur only at very narrow ranges of concentration and *p*H. The *p*H effect (fatty acids active only below *p*H 5; about 4.5) is rare enough but is of no therapeutic value since the activity is lost at physiologic *p*H's. Since our studies were carried out at physiologic *p*H, the data are not directly comparable.

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# The Unreliability of the Titan Yellow Method for the Determination of Magnesium in Patients Receiving Intravenous Calcium Gluconate

*Constantine S. Anast*

**Gluconate was found to interfere with the determination of magnesium by the Titan yellow method. With this method, false low results were found in the serum and urine of patients receiving intravenous calcium gluconate. The interference can be prevented by ashing of the specimen.**

CALCIUM GLUCONATE is frequently administered in clinical and experimental circumstances in which magnesium metabolism is being studied. Calcium gluconate has been used to study the effects of calcium on blood levels and renal excretion of magnesium (*1, 2*). It is commonly administered in patients with conditions in which there may be significant aberrations in magnesium metabolism, including electrolyte problems, alcoholism with delirium tremens, hypoparathyroidism, and renal disease.

The Titan yellow method is one of the most commonly used laboratory procedures for the determination of magnesium in body fluids. While studying newborn infants undergoing exchange transfusions for erythroblastosis fetalis, we found that gluconate markedly interfered with the determination of magnesium by the Titan yellow method. The phosphate precipitation method for the determination of mag-

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nesium was employed to evaluate results obtained by the Titan yellow method. Studies with both methods in a variety of clinical and experimental situations revealed that the presence of gluconate caused false low results in magnesium determination by the Titan yellow method.

## Methods

### Titan Yellow Method

The procedure followed is a modification of the method of Orange and Rhein (*3*). The reaction consists of the combining of magnesium hydroxide with the dye Titan yellow (Clayton yellow, National Aniline) in the presence of the dispersing agent, polyvinyl alcohol. The magnesium hydroxide–Titan yellow lake results in a pink color, the intensity of which is measured spectrophotometrically. The method was modified to determine magnesium concentrations in 0.2 ml. of serum. Urine samples were treated in the same manner, with the quantity of urine used being proportional to the estimated magnesium concentration.

### Phosphate Precipitation Method

The procedure employed is that of Dennis, as modified in the text of Hawk *et al.* (*4*). Ammonium oxalate is added to the serum to precipitate calcium. The magnesium in the supernatant solution is then precipitated as magnesium ammonium phosphate, and the latter is estimated by a colorimetric phosphate method. The magnesium content is then calculated from the determined phosphorus content of the precipitate. Urine samples are analyzed in the same manner, except that the calcium is precipitated at a *p*H between 4.2 and 4.6.

Determinations of magnesium were made by both methods on ten different samples of serum obtained from patients not receiving any medication. The agreement of results obtained by the two methods was always within 6% and the average difference was 3.2%.

## Results

### In-vitro Studies

Small amounts of calcium gluconate, when added to serum or to solutions of known magnesium concentrations, interfered with the magnesium–Titan yellow color development, with consequent false low results (Table 1). Five hundred micrograms (1.12 $\mu$M) of calcium gluconate resulted in 80% inhibition of the color development of 2 $\mu$g.

**Table 1.** Interference with the Titan Yellow Method by Calcium Gluconate when Added to Serum

| *Specimen* | *Magnesium (mg./100 ml.)* | |
|---|---|---|
| | *Titan yellow* | *Phosphate precipitation* |
| 5 ml. pooled serum + 0.1 ml. water | 2.04 | 1.90 |
| 5 ml. pooled serum + 0.1 ml. 10% calcium gluconate (0.9 mg. Ca) | 0.50 | 1.93 |
| 5 ml. pooled serum + 0.1 ml. 3.3% calcium chloride (0.9 mg. Ca) | 2.00 | |

(.08 $\mu$M) of magnesium with Titan yellow, while there was no significant inhibition by 10 $\mu$g. (.02 $\mu$M). Varying degrees of inhibition were found within these extremes (Fig. 1). Similar results were obtained with equivalent amounts of gluconate in the form of sodium gluconate. Equivalent amounts of calcium as calcium chloride did not result in significant interference (Table 1). At no time did calcium gluconate interfere with the determination of magnesium by the phosphate precipitation method.

The possibility that the dispersing agent or the particular brand of dye being used might be a factor was investigated. There was no change in the amount of inhibition when a different brand of dye (Titan yellow, Eastman Organic Chemicals) was employed. When

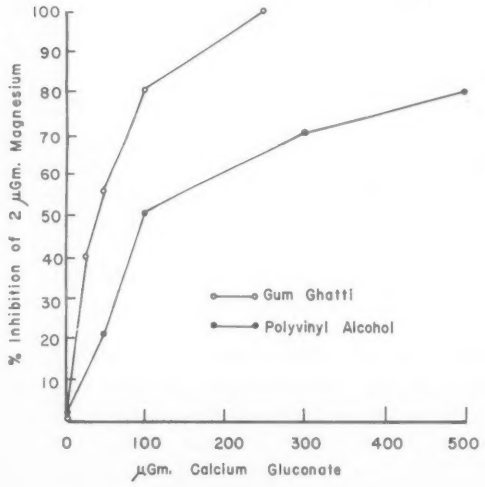


**Fig. 1.** Inhibition in estimation of magnesium concentration with Titan yellow by various amounts of calcium gluconate, using two different dispersing agents.

gum ghatti was substituted for polyvinyl alcohol as a dispersing agent, the percentage inhibition by a given amount of calcium gluconate was greater, so that only 250 $\mu$g. (0.56 $\mu$M) resulted in complete inhibition of color development with 2 $\mu$g. (0.08 $\mu$M) of magnesium (Fig. 1).

The interference by gluconate could not be equated on a molar basis with either magnesium or Titan yellow. With gluconate-to-magnesium ratios of 120:1 and 60:1, using polyvinyl alcohol as a dispersing agent, the percentage inhibition was 62 and 53%, respectively. Doubling the concentration of Titan yellow did not appreciably change the amount of inhibition.

### Determination of Serum Magnesium Levels in Infants Undergoing Exchange Transfusions

Infants with erythroblastosis fetalis received exchange transfusions of 500 ml. of citrated blood, with the administration of 1 ml. of 10% calcium gluconate after each 100 ml. of exchange. In 2 infants in whom determinations were made after each 100 ml. of exchange, a stepwise decrease to 60% of the original value was indicated by the Titan yellow method, while only mild fluctuations were found by phosphate precipitation. In 5 other infants, determinations were done before and immediately after the complete transfusion. By the Titan yellow method, substantially lower levels were apparent following the exchange in all 5 infants (average decrease, 39%). The phosphate precipitation method revealed no difference, or a slight lowering (average decrease, 6.6% —Table 2). Before the exchange, the average difference in values found by the two methods was 3.3%. Following the exchange, the values obtained by the Titan yellow averaged 34% lower than those obtained by phosphate precipitation. The sodium citrate–citric acid of the transfused blood was not found to be a contributing factor in the false low magnesium levels found by Titan yellow.

### Effect of Intravenous Administration of Calcium Gluconate on the Determination of Serum Magnesium

Employing the Titan yellow and phosphate precipitation methods, the effect of a single intravenous dose of 10% calcium gluconate administered over a 10-min. period was studied in 2 infants and 1 adult. No significant changes were found at any time by the phosphate precipitation method. However, significantly lower levels were found by

**Table 2.** Serum Magnesium Levels by Titan Yellow and Phosphate Precipitation Methods in Newborn Infants Before and Immediately Following Exchange Transfusions*

| | *Serum magnesium (mg./100 ml.) Titan yellow* | | *Serum magnesium (mg./100 ml.) Phosphate precipitation* | |
|---|---|---|---|---|
| *Infant* | *Before exchange* | *Following exchange* | *Before exchange* | *Following exchange* |
| 1 | 2.81 | 1.67 | 2.67 | 2.70 |
| 2 | 1.78 | 1.02 | 1.72 | 1.52 |
| 3 | 1.85 | 1.20 | 1.91 | 1.83 |
| 4 | 2.13 | 1.22 | 2.20 | 1.88 |
| 5 | 2.17 | 1.46 | 2.18 | 2.02 |

*Each infant received 5.0 ml. of 10% calcium gluconate intravenously during the procedure.

the Titan yellow method 10 min. following the injection (Table 3). In the adult, where serial determinations were done over a 2-hr. period, consistently lower levels were indicated by the Titan yellow method.

To demonstrate the degree to which a clinician could be misled if the Titan yellow method were used to determine serum magnesium concentrations of a patient receiving intravenous fluid therapy, a study was carried out in a 50-kg. woman who received 80 ml. of 10% calcium gluconate daily in a continuously flowing infusion. The apparent serum magnesium levels on 4 different days, as determined by Titan yellow, averaged 30% lower than those obtained by phosphate precipitation. The levels indicated by Titan yellow would be considered pathologically low, while those found by phosphate precipitation were within the low normal range.

**Table 3.** Serum Magnesium Levels by Titan Yellow and Phosphate Precipitation Methods Before and After the Intravenous Administration of Calcium Gluconate

| | | | *Serum magnesium levels (mg./100 ml.)* | | | |
|---|---|---|---|---|---|---|
| | | | | *Minutes following injection* | | |
| *Patient* | *Calcium gluconate (ml. of 10% solution)* | *Method* | *Before injection* | *10* | *30* | *120* |
| 1 (infant) | 8.0 | Titan yellow | 1.82 | 1.36 | | 1.89 |
| | | Phosphate precipitation | 1.76 | 1.82 | | |
| 2 (infant) | 5.0 | Titan yellow | 1.92 | 1.28 | | 1.83 |
| | | Phosphate precipitation | 1.90 | 1.83 | | |
| 3 (adult) | 30.0 | Titan yellow | 2.15 | 1.66 | 1.81 | 1.94 |
| | | Phosphate precipitation | 2.19 | 2.06 | 2.17 | 2.16 |

### Effect of Intravenous Administration of Calcium Gluconate on the Determination of Magnesium in the Urine and Serum Studied Simultaneously

Because urinary magnesium concentrations are also determined by the Titan yellow method in some laboratories, it was of interest to know whether these results also would be invalidated by gluconate infusion. Therefore, serial determinations of serum and urine magnesium levels were made in a 54-kg. woman who received 65 ml. of 10% calcium gluconate in 500 ml. of 5% glucose over a 90-min. period. Results of the measurements of serum magnesium were as expected: During the infusion (at 60 min.) the serum magnesium level indicated by Titan yellow was 45% lower than that found by phosphate precipitation. Seventy-five minutes and 200 minutes following the infusion, the apparent serum levels by Titan yellow were 34 and 15% lower than the values obtained by phosphate precipitation.

During and after the same infusion, urinary magnesium concentration, when measured by Titan yellow, appeared to decrease; while by phosphate precipitation, there was a striking increase in urinary magnesium concentration (Fig. 2). The greatest disparity was found 75 min. after the infusion, when the concentration determined by phosphate precipitation was 40 times that obtained by Titan yellow. Two hundred minutes following the infusion, the concentration determined by phosphate precipitation was still 6 times the level indicated by the Titan yellow method. When aliquots of urine were ashed to destroy gluconate, the concentrations found by the Titan yellow method were almost identical to those obtained by the phosphate precipitation method (Fig. 2).

Similar results were found in a 25-kg. boy who received 32 ml. of 10% calcium gluconate over a 4-hr. period. Serum values in milligrams per 100 ml. by phosphate precipitation and Titan yellow methods respectively were: before infusion, 1.77 and 1.81; at completion of 4-hr. infusion, 1.64 and 1.24; 4 hr. following completion of infusion, 1.75 and 1.70. Total urinary excretion of magnesium over the 8-hr. period was 12 mg. by Titan yellow, 31.2 mg. by Titan yellow preceded by ashing of specimen, and 30.2 mg. by phosphate precipitation. It can be seen that 60% of the urinary magnesium was not detected by Titan yellow unless the specimen was previously ashed.

## Discussion

It is apparent that gluconate in some manner interferes with the determination of magnesium by the Titan yellow method. Our concern

has been more with the clinical and investigative implications of this interference than with its chemical nature. Calcium gluconate is a commonly used medication. It is frequently administered intravenously under conditions in which magnesium metabolism is being studied. It is important, therefore, to be aware of the false low results that gluconate can introduce if magnesium is estimated by the Titan yellow

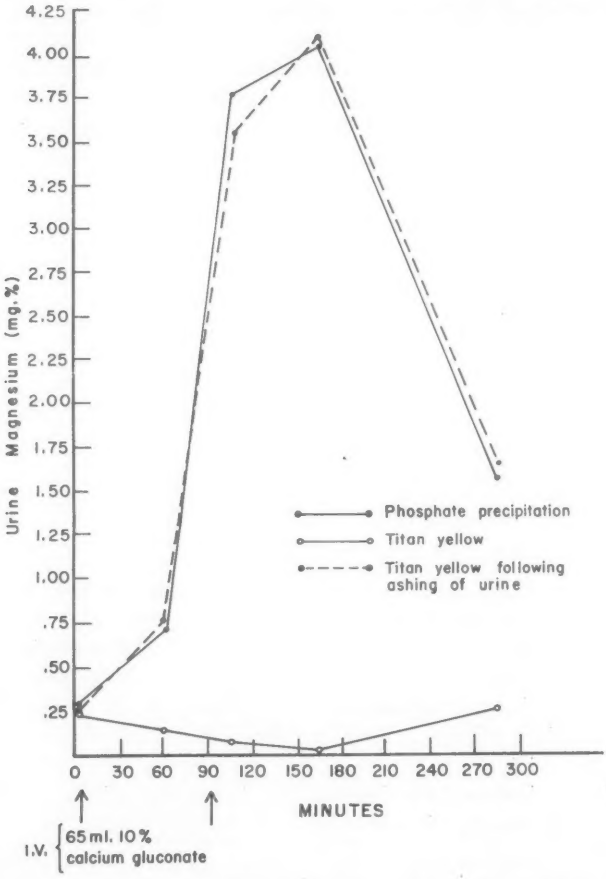


**Fig. 2.** Urine magnesium levels as determined by Titan yellow and phosphate precipitation methods in a 54-kg. woman who received 65 ml. of 10% calcium gluconate intravenously in 500 ml. of 5% glucose over a 90-min. period.

method. The percentage inhibition increases with increasing concentrations of gluconate. Therefore, from a clinical standpoint, important factors are: size of the patient, dose of calcium gluconate administered, and the rate at which it is metabolized and excreted.

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# A Stable "Bilirubin" Standard

*P. K. Bilissis and R. J. Speer*

**An aqueous solution of N-(1-naphthyl) ethylenediamine dihydrochloride has been successfully employed as a routine secondary "bilirubin" standard. It possesses a stability which is, unfortunately, not a characteristic of bilirubin itself.**

THE ADVENT of automation for the daily determination of bilirubin in many serum samples has intensified our need for stable bilirubin standards for routine, repetitive calibration purposes. Crystalline bilirubin serves as a suitable primary standard, and when dissolved in chloroform, it yields reasonably stable stock solutions. However, when this is diluted with methanol to prepare a working standard, the resulting solution lacks adequate stability for routine daily use. Similarly, when bilirubin is dissolved in dilute alkali and quickly diluted with albumin solutions, the working standards are not as stable as one might desire. Some authors have suggested the use of pooled serum specimens or of commercial quality control sera, but these are neither as convenient nor as economical as one might like. In seeking a solution to this prosaic but practical problem, we have sought a pure, stable, commercially available aromatic amine (or related material) which would couple with diazotized sulfanilic acid under the appropriate reaction conditions to yield a chromogen with spectral qualities similar to those of azobilirubin. Such a material, N-(1-naphthyl) ethylenediamine dihydrochloride, has been found, and its applicability as a simulated bilirubin standard has been proved during the past 18 months of daily routine use.

## Experimental

*Concentrated Stock Solution* Prepared by dissolving 129 mg. of N-(1-naphthyl) ethylenediamine dihydrochloride in sufficient distilled

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water to make 200 ml. This solution is stable for many months when stored in a brown bottle at 4°.

*Simulated "Bilirubin" Working Standards* Distilled water is employed to dilute the concentrated stock solution (5.0 ml. stock diluted to 100 ml. is approximately equivalent to 5 mg./100 ml. bilirubin; 10.0 ml. stock diluted to 100 ml., approximately 10 mg./100 ml. etc.)

These solutions should be stored in brown polyethylene dispenser bottles at 4° when not in actual use and are stable for at least 2 months. The apparent bilirubin concentration of each should be checked against a freshly prepared bilirubin standard with the specific analytical procedure and the particular spectrophotometer for which they are to be employed prior to their use as daily secondary standards.

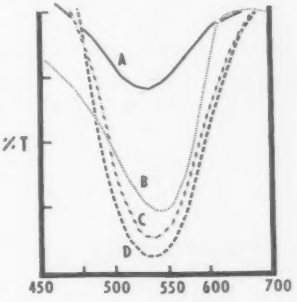


**Fig. 1.** Pigments derived from coupling diazotized sulfanilic acid with *A*, crystalline bilirubin (in weak alkali diluted with albumin solution); *B*, N-(1-naphthyl)ethylenediamine dihydrochloride solution; *C*, Versatol Pediatric (Warner-Chilcott); and *D*, icteric human serum.

## Spectral Characteristics

Figure 1 illustrates the close correspondence between the λ max of authentic azobilirubin in serum and that of the azo dye derived from N-(1-naphthyl) ethylenediamine dihydrochloride. These characteristics have permitted the use of this simulated standard in the Beckman Model B, Beckman Model DK-2, Spinco Ultramicrophotometer,* and the Technicon Autoanalyzer† on a routine basis for the past 18 months.

*Beckman Instruments, Inc., Spinco Division, Palo Alto, Calif.
†Technicon Instrument Corp., Chauncey, N. Y.

# Modification of the Fluorescence Attachment for the Beckman Model DU Spectrophotometer

*Morris Rockenmacher and Andrew F. Farr*

**Details are given for the modification of the fluorescence attachment for the Beckman DU instrument so that all filters are easily changed. The versatility of the instrument is improved as the attachment does not have to be removed when the instrument is used as a spectrophotometer.**

THE FOLLOWING describes a relatively simple and inexpensive modification of the Beckman No. 2980 fluorescence attachment for the Beckman DU spectrophotometer which permits rapid and easy interchange of both primary and secondary filters and increases the flexibility and adaptability of this instrument in fluorimetric analysis.

The filter plate assembly for the secondary filters as originally supplied with the fluorescence attachment (Fig. 1, upper half) can be fitted with a maximum of two filters. Changing of these filters in the standard attachment involves the removal of the filter plate from the spectrophotometer photo tube housing and disassembly of the filter plate.

The authors' modifications of the secondary filter plate and filter carrier (Fig. 1, lower half) were accomplished after removal from the photo tube housing and disassembly. The top and flat spring portion of the filter carrier were sawed off (Fig. 1, at center left). The tops of the rectangular filter compartments of the modified filter carrier were left open. A spring-loaded friction contact was added at the top of the center vertical pillar between the filter compartments to maintain the vertical orientation of the filter carrier. The spring-

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loaded contact replaced and served the same purpose as the flat spring in the original filter carrier. Light metal filter frames for holding the secondary filters were made from angle brass to fit into the filter compartments. An "eye" at the top of this frame through which a hook

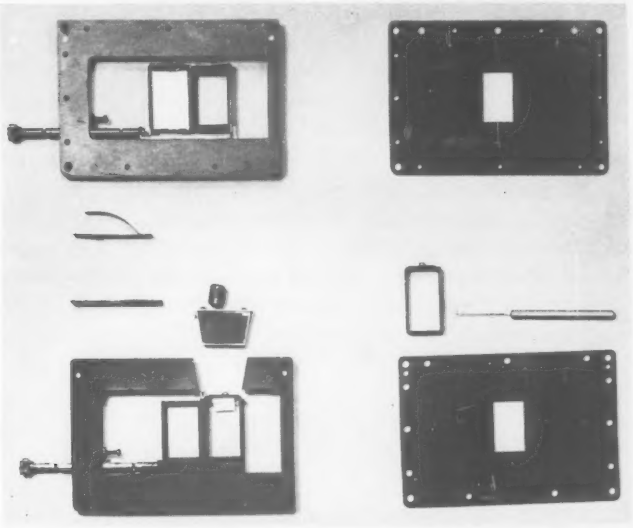

**Fig. 1.** Filter plate assembly and modifications (see text).

(Fig. 1, at right center) can be passed, allows the filters to be lifted out of the carrier through an opening made in the filter plate frame as described below. The sheet-metal filter frames are not essential if the filters are dimensioned to fit and black plastic electrician's tape used as a border and upper tab. A hole in the tab accommodates the hook for manipulation of the filters.

The filter plate frame was modified by cutting, from the top, a trapezoidal section large enough to allow passage of a filter (Fig. 1, lower left). A slot ⅛ in. wide by ¼ in. deep was also milled into the top of the frame to provide space for a latch. A trapezoidal plug was constructed of brass to fit into the cut-away section of the frame. A thin, sheet-brass rectangle slightly larger than the top of the plug was installed on the plug to eliminate light leaks. A sliding tongue fitted beneath the brass rectangle and attached to a projecting knob can be moved into

the milled slot as a latch. After these alterations, the filter plate was reassembled and reinstalled on the photo tube housing of the spectrophotometer. The modified secondary filter assembly provided a filterless position (knob completely in) and two filter positions (knob out to first or second detent grooves). Secondary filters were interchanged by pulling the filter positioning knob out to the first or second detent grooves, disengaging the sliding tongue bar and lifting off the trapezoidal cover. The filters were interchanged by means of the hook, and the cover was replaced and secured with the sliding tongue bar.

Alteration of the instrument to provide interchangeability of primary filters requires no machine work and is achieved by separation of the cuvet carrier housing from the cell compartment, removal of the primary filter slide, removal of the primary filter disc, replacement of primary filter slide (minus filters), and reassembly of the units. Primary filters of desired thickness and wave-length transmission properties were ordered with dimensions of $15 \times 32$ mm. In operation, the selected primary filter is positioned on the floor of the cell compartment housing over the circular opening for the exciter light source and under the sheet-metal light mask with the diagonal bar-shaped slot. This can be done easily with the fingers or a pair of curved forceps after removal of the cuvet carrier from the housing.

The modified instrument has proved to be satisfactory in over 18 months of service in this laboratory. Furthermore, the modified fluorescence attachment may be left in place for spectrophotometric measurements. By replacing the fluorescence attachment tungsten lamp backplate assembly on the lamp housing with the standard (spectrophotometric) tungsten backplate assembly and pushing the secondary filter positioning knob to the blank (closed) position spectrophotometry may be performed in the usual fashion. This dual application of the modified attachment for spectrophotometry and fluorimetry is time-saving and convenient. The described alterations extend the usefulness of the instrument by making possible easy and convenient interchange of a wide selection of primary and secondary filters for fluorimetry.

# Use of a Lyophilized Serum Enzyme Preparation in Quality Control

*Melford Q. Hersey, Katherine Hartwell, and Richard P. Doe*

**A purified preparation of alkaline phosphatase added to standardized lyophilized serum has been used by the Minneapolis Veterans Administration Reference Laboratory to evaluate the routine alkaline phosphatase method in use in the 10 laboratories in its reference area.**

**Evidence of a marked need for investigation of the routine method for alkaline phosphatase determination was found in 3 of the 10 laboratories.**

**A semiquantitative phosphatase method proved to be useful as an independent procedure for use in evaluation of the different laboratories.**

A CONTINUING NEED exists for better and extended quality control in the clinical laboratory. Surveys such as those conducted by Belk and Sunderman (*2*), Wootton and King (*17*), Wootton (*16*), Snavely *et al.* (*14*), and more recently a review by Freier and Raush (*8*) on the measures available to insure the reliability of clinical chemistry results, all emphasize this need. There is a disagreement among some workers (*5*, *15*) as to whether the use of a frozen pooled serum for a control in determining alkaline phosphatase activity is sufficient for this purpose. Chemical standards likewise do not always fulfill the criterion for quality control measures, but they do serve as a guide in certain areas where pooled serum is inadequate. Recent advances in the use of commercially prepared normal and abnormal sera preparations* containing varying amounts of the various blood constituents have eliminated some problems arising in the collection of a pool of serum used as a quality control measure. The latest addition to the increasing list of

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The Phosphatabs used in this study were kindly supplied by Mr. Stephen Koziol, General Diagnostics Division, Warner-Chilcott Laboratories, Morris Plains, N. J.

*Versatol, Labtrol, Hyland Control Serum, Pathrol, etc.

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commercially prepared materials intended to serve as control is a purified enzyme preparation, alkaline phosphatase,* incorporated into lyophilized serum which has been standardized against such factors as would interfere with the determination of alkaline phosphatase activity. Its use as a means of checking the accuracy of several methods for measuring alkaline phosphatase activity was studied.

A study of alkaline phosphatase results obtained on the same material by 10 different clinical laboratories in a five-state area of the Veterans Administration has been made by the Reference Laboratory of that area. The purpose of this study was to determine the range of values which might be expected when a single sample was analyzed by a number of different laboratories, each using their routine method of alkaline phosphatase analysis, and also using a new simplified tablet screening method (Phosphatabs) (*1, 6, 13*). The results from these laboratories were used to evaluate the usefulness of the lyophilized standard as a quality control check for a specific enzyme procedure. Individual reports of duplicate determinations of alkaline phosphatase by the method in regular use in each laboratory were requested, as well as duplicate reports on the same lyophilized material using the screening test. Reports were received within a period of 6 weeks. Three laboratories made only a single report for each method.

## Methods and Materials

The material supplied to each laboratory by air mail consisted of six vials of lyophilized serum with an added lyophilized beef kidney alkaline phosphatase preparation representing four concentrations of alkaline phosphatase.* These were prepared from pooled normal human serum to which alkaline phosphatase extracted from beef kidney by the method of Morton (*11*) and having an optimum *p*H of 9.6 had been added in varying amounts. Each vial was to be reconstituted shortly before analysis by the addition of 5 ml. of distilled water. All of the variable factors which might influence a particular assay, such as *p*H, magnesium, phosphate, and protein concentration, were identical in all specimens. A box of tablets for the semiquantitative screening procedure, with directions for conducting this test, was also provided.

The screening test was read by comparing the color developed in controlled-diameter tubes with the routine color chart. Special charts

*Warner-Chilcott Laboratories, General Diagnostics Division.

**Table 1.** THE CALCULATED VALUE FOR THE ENZYME STANDARD, THE BODANSKY VALUE OBTAINED BY THE WARNER-CHILCOTT LABORATORY, AND THE KING-ARMSTRONG VALUE OBTAINED BY THE REFERENCE LABORATORY

| | *Alkaline phosphatase activities (KAU)* | | | |
|---|---|---|---|---|
| | *Specimens I & II* | *Specimens III & IV* | *Specimen V* | *Specimen VI* |
| Calculated value | 14.8 | 25.6 | 5.8 | 47.4 |
| Warner-Chilcott Laboratory (Bodansky*) | 14.8 | 26.4 | 5.6 | 44.8 |
| Reference Laboratory (King-Armstrong) | 15.0 | 26.4 | 5.1 | 46.0 |

*A factor of two was used to convert Bodansky units to King-Armstrong units. The crude alkaline phosphatase preparation contained about 0.48 K.A.U./mg. and this factor was used to obtain the calculated value.

for icteric and very icteric sera were included. The four colors on the chart corresponded to alkaline phosphatase levels designated normal (6–12 KAU%),* borderline (12–24 KAU%), elevated (24–44 KAU%), and highly elevated (above 44 KAU%).

The six vials which were supplied to each participating laboratory included two sets of identical material, thus affording two hidden duplicates in addition to the duplicate determinations requested on each vial. The numerical order of the specimen numbers as sent out alternated the hidden duplicates of the two concentrations. The duplicated specimens were of borderline and elevated levels. The single specimens included sera with normal and highly elevated levels. Two sets of specimens were run at different times and by different people in the Reference Laboratory.

## Results and Discussion

To provide a basis of comparison for the different methods, any reports made in Bodansky units (including the pre-analysis figure) were converted to King-Armstrong units by multiplying by a factor of 2. Confirmation of this factor was found by comparing the control values to the results found by the Reference Laboratory; this comparison is shown in Table 1. These results, expressed in the same units (KAU), are in close agreement with each other and also the calculated values. Figures 1–4 illustrate the values obtained for each of the four concentrations of alkaline phosphatase given out. These show all results reported in terms of King-Armstrong units, with duplicates appearing in the same vertical column. The heavy line represents the mean value

*KAU represents King-Armstrong Units.

of the specimen. This value was obtained by the Reference Laboratory and is the value upon which the comparison of results received from other laboratories was made. The screening test results are indicated by the first letter of the range in which the reading was made, with the

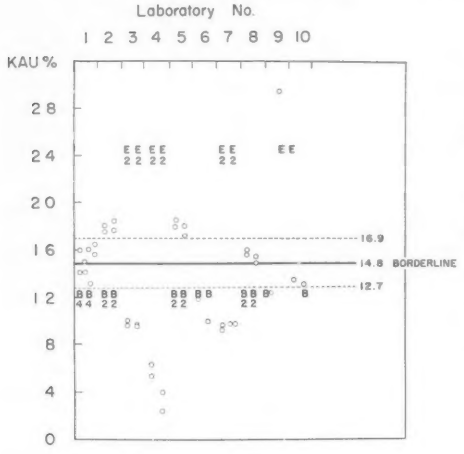


**Fig. 1.** Results on Specimens I and II from Minneapolis V.A. Reference Laboratory (Laboratory 1) and 9 participating area laboratories for the screening method (B indicates borderline, 12–24 KAU%; E indicates elevated, 24–44 KAU%) and from their routine method (°). Heavy line represents correct value for control material and dotted lines represent three standard deviations. All errors in screening method were to the high side whereas errors from regular method in use were scattered about mean.

number of determinations included below. Where no number is used, only a single report was received.

Confidence limits have been calculated based on the determinations made in this study by the Reference Laboratory where the greatest number of duplicates were run. The average difference between duplicates ($\overline{R}$) was 1.7 KAU. The mean of all determinations was 23.1 KAU. Confidence limits include three standard deviations as defined by the formula (*12*): Mean $\pm$ 1.88 ($\overline{R}$). Thus the mean value of 23.1 $\pm$ 3.2 includes three standard deviations, and this represents a variation of almost 14% of the mean. Applying the percentage figure to the Reference Laboratory means for the four levels of alkaline phosphatase concentrations, the 99% confidence limits of Specimens I and II would be 12.9 to 17.1; of III and IV, 22.7 to 30.1; of V, 4.4 to 5.8; and of VI, 39.6 to 52.4 KAU. These are indicated on the figures by dotted lines.

Figure 1 illustrates the range of values received on duplicate Specimens I and II. The assigned screening value for this phosphatase level is borderline (12–24 KAU%). Reports by this method showed 63% to be within the assigned values, while 37% of the results were above the

upper level. No results were below 12 KAU% by the screening method. By the regular routine methods used in the various laboratories 37% of the values reported were with 99% confidence limits of 12.9–17.1 KAU% set by the Reference Laboratory. However, of the 63% of the

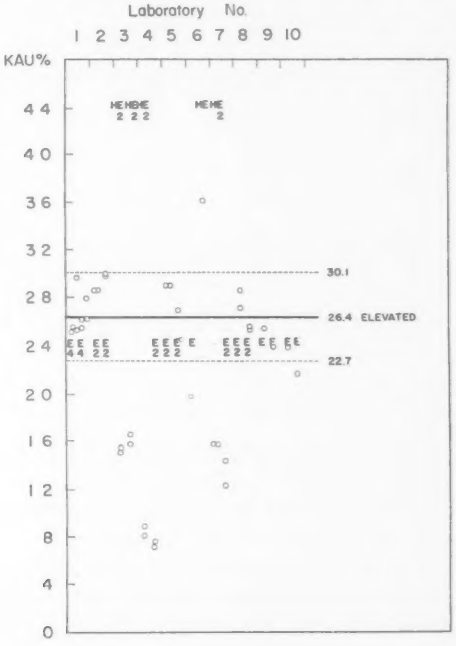


**Fig. 2.** Results from control Specimens III and IV using same scheme as in Fig. 1. Again main errors were falsely normal values with routine method in use and falsely elevated values with screening method.

results not within the confidence limits, 39% were below the lower limit. This is in contrast to the findings of the screening test. In this instance the semiquantitative method would be preferred over the regular method in use since no false normal values were given. The tendency to occasionally report the screening value as too high was noted throughout the study. This resulted from attempts to read the color reaction to the nearest color standard rather than waiting until the color reached the next higher color standard before putting the value in the higher range. Carefully worded instructions would eliminate most of this error.

Figure 2 illustrates the range of values received on Specimens III and IV. The mean of these specimens is 26.4 KAU% and the 99% con-

fidence limit is 22.7–30.1 KAU%. Sixty per cent of the results by regular routine methods were within these confidence limits whereas 37% of the 40% without the confidence limits fell below the lower limit. These low results were again false normal values. The screening value

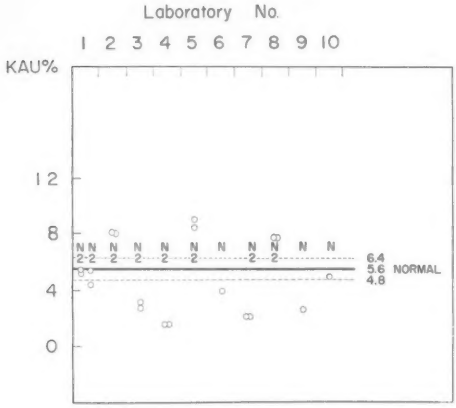


**Fig. 3.** Results from control Specimen V which was in normal range using same scheme as in Fig. 1. Screening results were correct in all instances whereas errors from regular method in use were scattered about mean.

assigned to these specimens was 24–44 KAU%. There were no values reported below 24 KAU%. Seventy-six per cent fell within the assigned range and 24% were reported as highly elevated. No false normals were reported by this method.

Figure 3 illustrates the range of values received on Specimen V. The mean of this specimen was 5.1 KAU%; the 99% confidence limits were 4.4–5.7 KAU%. Only 26% of the reports by the regular routine methods were within the confidence limits. Forty-two per cent of the values were below the lower limit and 32% were above the upper limit. However, the screening test reports were all within the assigned normal rang of 6–12 KAU%.

Figure 4 illustrates the range of values reported on Specimen VI. The screening range assigned to this specimen was highly elevated—44 KAU% and above. All but one report fell within the assigned range; the single report was read in the next lower assigned range, elevated. On the other hand, 42% of the results by the regular routine method fell below the 99% confidence limits of 39.6–52.4 KAU% set by the Reference Laboratory. One report (5%) was above the upper limit. Only half (53%) of the results of the routine procedures were within the confidence limits.

It is obvious from the figures that the reports from different laboratories varied widely. The spread in the quantitative assay varied from five-fold in Specimens VI (10.8–53.8 KAU/100 ml. reported) to twelve-fold in Specimens I and II (2.4–29.4 KAU/100 ml. reported). In general, there was as much variation between duplicate analyses on a single vial as there was between the average analyses of the hidden duplicate vials, indicating an unbiased reporting of the data. Duplicates were rarely more than 3 KAU apart. In 15 instances there was a marked discrepancy between duplicate results on the same material by the two methods employed. In all but one of these the correlation between duplicates was close.

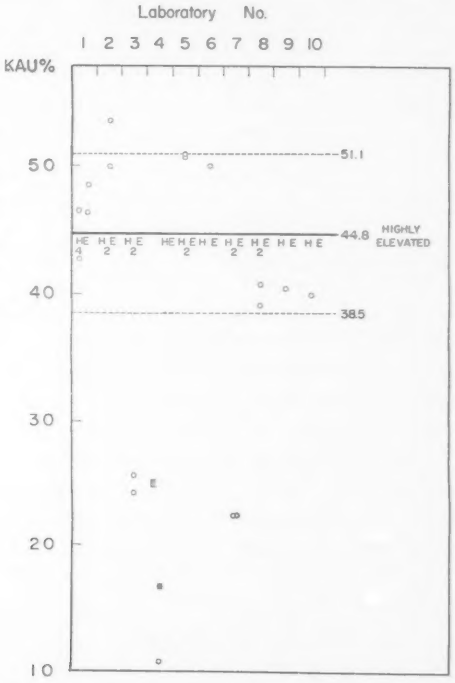


**Fig. 4.** Results from control Specimen VI which was in highly elevated (*HE*) range using same scheme as in Fig. 1. All screening results were in correct range except for one elevated (*E*) report, whereas many values from routine method were below acceptable range.

Expressed in the same unit value the results of two methods applied to the same specimen should be in very close agreement. The 15 times when this was not true in this study represents results from but three different laboratories, giving a strong indication that inaccurate re-

**Table 2.** Comparison of Results Reported by the Routine Alkaline Phosphatase Method and the Screening Method

| *Specimen No.* | *Range of values arbitrarily assigned to screening test* | *Regular routine method** | | | *Screening method** | | |
|---|---|---|---|---|---|---|---|
| | | *Above* | *Within* | *Below* | *Above* | *Within* | *Below* |
| Specimens I & II | Borderline (12-24 KAU%) | 1 (3) | 23 (60) | 14 (37) | 14 (37) | 24 (63) | 0 (0) |
| Specimens III & IV | Elevated (24-44 KAU%) | 0 (0) | 24 (63) | 14 (37) | 9 (24) | 29 (76) | 0 (0) |
| Specimen V | Normal (6-12 KAU%) | 0 (0) | 6 (32) | 13 (68) | 0 (0) | 19 (100) | 0 (0) |
| Specimen VI | Highly elevated (44 KAU% & higher) | 0 (0) | 8 (42) | 11 (58) | 0 (0) | 18 (95) | 1 (5) |
| TOTALS | | 29 (25) | 61 (54) | 24 (21) | 23 (20) | 90 (79) | 1 (1) |

*Number and percentage (in parentheses) of tests by each method falling within or without the correct screening range.

ports are coming from their routine method and that investigative procedures to improve the control results are needed in those areas. A laboratory can measure its own precision in a procedure by repeated analyses of a single sample and by use of two independent assay methods to check the accuracy of either of the assays.

All results on the same material were averaged for the comparison summary in Table 2, which shows the number of times the routine method would have fallen in the correct screening range as compared to the number of times the screening values resulted in selection of the correct range. The value from the regular method was invariably within the correct range or below it. On only one occasion was the regular method too high. On the other hand, the screening value was either within the correct range or above it. As previously noted, even the few falsely high screening values would probably be read in the correct range if the technologist waited until the sample color reached the next higher color standard rather than reading it to the nearest color standard. It would appear that laboratories which have not instituted quality control procedures might obtain more accurate enzyme assays, although not as precise, using a simplified method such as the noted screening method in which the many factors which can influence the enzyme activity are easier to control.

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# Metabolic Disorders Leading to Mental Deficiency

## II. Screening for Excessive Urinary Excretion of Nonurea Organic Carbon and Nitrogen

*J. L. Karlsson*

**A new approach is described for the detection of abnormal metabolites in the urine of patients with known or suspected metabolic disorders. Distribution curves are presented for nonurea organic carbon and nitrogen which form a basis for judging whether grossly abnormal amounts of organic materials are present in the urine.**

**Families with recurrent mental retardation have been identified whose mental defect may be on an unknown metabolic basis.**

PRECISE METHODS have been developed for chemical studies of genetic disorders in microorganisms. The initial steps usually depend on growth trials and are not applicable to man (*1*). Human biochemical disorders have generally been discovered in the course of nonspecific studies or by screening methods differing entirely from those used in microbiology. The most extensively employed chemical screening procedures have been those for reducing substances—proteins, amino acids, indoles, and phenols—in the urine (*2–5*).

It is now generally felt that metabolic disorders are more frequent than was previously realized and that a new era of medicine lies ahead in the discovery of the precise chemical etiology of hereditary diseases. Increased understanding in this area promises to lead to specific therapy in many disorders which at present are handled symptomatically.

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It is believed by many authorities that besides the rare, recessively inherited metabolic disorders, diseases such as schizophrenia (*6, 7*), hypertension, and allergic tendency (*8*) may eventually be found to have a metabolic basis.

The most pressing need for research in this field is the development of improved methods which will make the detection of biochemical abnormalities possible at the clinical level. The present paper describes a procedure designed to detect gross abnormalities in urinary excretion of various metabolites. It is hoped that further refinement will be possible, but in itself it serves the purpose in excluding or including gross abnormalities before more specific search is made for minor aberrations. Our methods are based on the knowledge that, with the exception of urea, the urinary excretion of organic compounds tends to be relatively constant and even more so if the diet can be controlled—at least, qualitatively. The excretion of nonurea organic carbon and nitrogen thus becomes an index of the presence or absence of abnormal metabolites. The normal excretion of nonurea metabolites is sufficiently large to obscure in this kind of study anything but a gross spillage of metabolic products. It is interesting, however, that our method seems capable of detecting many of the currently known metabolic diseases.

## Patient Material

Because of the nature of this institution, all the patients are diagnosed as mentally deficient. Our work, in essence, has been an attempt to discover hitherto unknown metabolic causes of mental defect, although the methods are generally applicable to all metabolic diseases. The patients included in the study were selected on the basis of there being two or more siblings admitted to this hospital, with at least one available for study, as this group is likely to have more numerous hereditary disorders. A total of 105 unrelated families fitting these criteria were available after families with multiple mongolism, known biochemical diseases, or siblings with definitely different disorders had been excluded. The total population of the hospital is almost 4000. Although the information on the parents is inadequate, it was estimated that one-fourth of the individuals came from parents of borderline or low mentality and one-half from normal parents; one-fourth could not be classified. The age distribution was as follows: 0–20 years, 85 individuals; 21-40 years, 67 individuals; above 40 years, 37 individuals. The distribution of intelligence quotients indicated that 55 indi-

viduals had I.Q. from 0–20, 93 from 21–50, and 41 above 50. The group included 122 males and 67 females, a total of 189 being available for study. The majority of the patients have no specific diagnosis other than mental deficiency, but a few cases of hereditary diseases such as primary microcephaly are included.

## Methods

In order to avoid unnecessary variables in this initial phase of the study, the patients were placed on a qualitatively standardized diet and admitted to a special ward with nursing personnel trained in the necessary procedures. For 3 days the diet was composed of ½–¾ skim-milk solids, and on the fourth day, only skim-milk products. Some of the patients in the second half of the group were on anticonvulsive or tranquillizing medications, but the total amount of organic material was calculated not to contribute significantly to the total urinary output. Urine was collected for 24 hours on the fourth day and stored in a refrigerator during the collection. After the total volume had been measured, a portion was centrifuged and stored frozen for subsequent analyses.

Most of the chemical methods have been described in a previous report (*9*). Total organic carbon was determined by the wet combustion method of Van Slyke and Folch (*10*), which proved well-suited for this purpose. As the *p*H of the urines was generally in the acid range, an adjustment to remove bicarbonate was not necessary. Results were not altered on many samples which were adjusted to *p*H 4. A measured quantity of urine, usually 0.25 or 0.5 ml., was evaporated to dryness on a steam bath in the combustion tube and stored in a desiccator until the total carbon was estimated. Total nitrogen was determined by the micro-Kjeldahl method. Nonurea organic carbon and nitrogen were obtained by subtraction.

## Results

Figure 1 shows the distribution of the daily output of nonurea organic nitrogen per unit body weight in relation to intelligence quotients, the latter obtained from the medical record. It is interesting that patients with an I.Q. below 40 seem to fall rather frequently above 60 mg. nonurea organic nitrogen per kilogram, whereas only 2 patients above this I.Q. level have values that high. However, the majority of the patients had low I.Q., and more work is required before significance

can be assigned to this finding. Figure 2 shows daily output of nonurea organic carbon, and Fig. 3, nonurea organic nitrogen as a frequency distribution. It will be noted that certain individuals are removed from the general trend. A curve similar to that of Fig. 3 has been reported

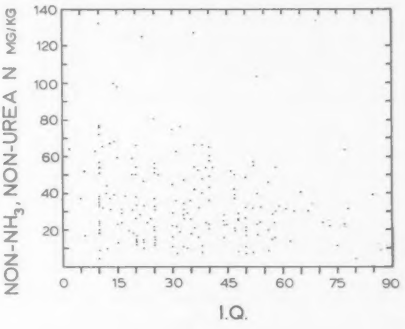



**Fig. 1.** Daily urinary excretion of nonurea organic nitrogen in relation to intelligence quotients.

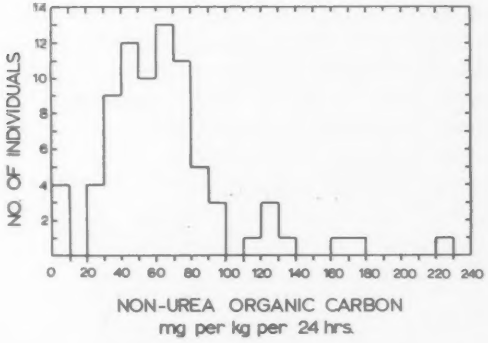



**Fig. 2.** Distribution of urinary excretion of nonurea organic carbon per unit body weight.

(*9*), but included only the first 50 families, whereas Fig. 3 includes all 189 individuals from 105 families. The general shape of the curve remains essentially unchanged. Figure 2 is limited to the members of the first 50 families on whom specimens were still available. Figures 4 and 5 express the same data in terms of creatinine in an attempt to correct for losses of urine which were unavoidable in a few cases. These last two figures may also be of value for comparison with data obtained on casual urine specimens. Figure 6 shows the distribution of organic acid excretion per unit body weight.

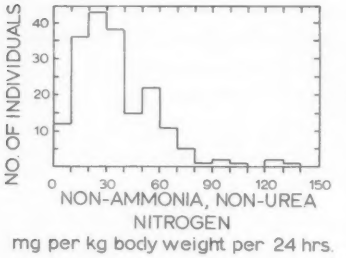
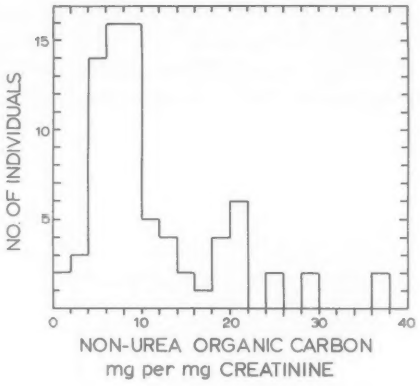
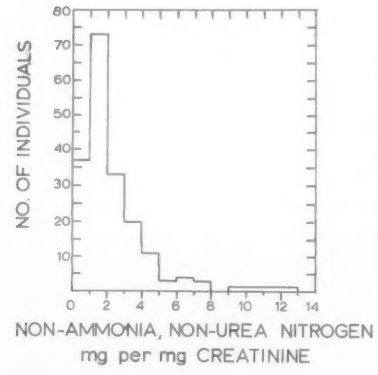



**Fig. 3** (*above*). Distribution of urinary excretion of nonurea organic nitrogen per unit body weight. Expected average is 30 mg./kg./day. **Fig. 4** (*right*). Distribution of urinary excretion of nonurea organic carbon per unit creatinine.




A recent paper by Dedmon *et al.* (*11*) describes a paper chromatographic screening procedure for organic acids, modified from the method of Nordmann (*12*) and designed to detect specific organic acids rather than a general increase. Of 10 patients with phenylketonuria studied for nonurea nitrogen, 8 were found to excrete over 60 mg./kg./day, the mean for the group being 63 mg./kg./day or twice that for the population in Fig. 3. Known metabolites in the urine of phenylketonuric patients are insufficient to account for the increase (*13*).



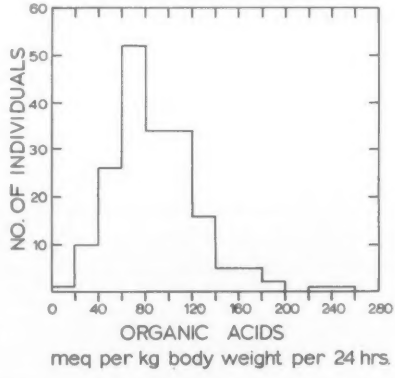




**Fig. 5** (*left*). Distribution of urinary excretion of nonurea organic nitrogen per unit creatinine. **Fig. 6** (*right*). Distribution of urinary organic acid excretion per unit body weight.

## Discussion

The data reported in this paper suggest that certain families have been encountered whose recurrent mental retardation may have a metabolic basis. Only further work will establish whether such is the case. It seems that values for nonurea organic nitrogen consistently above 60 mg./kg./day are abnormal under the conditions of this study. Some of the families with high nitrogen values also have high carbon values. It is hard to explain how organic nitrogen can be high without the carbon being elevated, and such discrepancies will be thoroughly scrutinized. At this time it is felt that a value for nonurea organic carbon exceeding 120 mg./kg./day is abnormal under the conditions of this study.

In general it has been found that other retarded siblings from a given family have high values on the same measures if one sibling is high. Some families have high values on all the parameters, and these are considered the prime "suspects" for further study. While most of the "suspects" carry no specific diagnosis, three families with primary microcephaly have shown high values on several tests. Work is in progress to attempt to identify chemical constituents responsible for the elevated carbon or nitrogen. It should be pointed out that none of the "suspects" has aminoaciduria.

The basic data presented in this paper make it possible to judge whether an individual is excreting grossly abnormal amounts of organic compounds. A study of a population with one disease would presumably make it possible to detect smaller increases by comparing the mean value with the mean for a control population.

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# A Modified Ninhydrin Colorimetric Method for the Determination of Plasma Alpha-Amino Nitrogen

*Lillian J. Fisher, Sylvia L. Bunting, and Leon E. Rosenberg*

**The present report describes the development of and results with a modified ninhydrin colorimetric method for the determination of free plasma α-amino nitrogen. Precipitation of the plasma proteins with a hydrochloric acid-sodium tungstate reagent, filtration of the deproteinized filtrate through glass wool plugs, and reduction of the *p*H of the reaction resulted in improved reproducibility, lower water blank readings, and lower plasma α-amino nitrogen concentrations which agreed closely with values obtained using other standard technics. The stoichiometry of the modified reaction and recovery of standard amino acid solutions were investigated and found to be satisfactory. A comparative study with the ninhydringasometric technic showed that the modified colorimetric procedure described herein yielded values in very close agreement with the more laborious manometric determination. Fasting venous plasma samples obtained from 21 normal volunteers yielded a mean plasma concentration of 3.85 mg./100 ml. The report concludes from these observations that the modified colorimetric procedure provides an accurate, simple, and rapid estimation of the total free amino acid content of human blood plasma.**

RECENT YEARS have witnessed a marked increase in interest in the clinical investigation of amino acid metabolism. Elegant column chromatographic technics for the accurate quantitation of individual amino acids in plasma and other body fluids have been developed (*1*, *2*), but an accurate, simple screening method for total plasma amino acid content has not appeared. Heretofore, the ninhydrin gasometric technic of Hamilton and Van Slyke (*3*) has been the only well-standardized assay for plasma alpha-amino nitrogen, but, despite its accuracy when properly performed, this method required special appara-

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tus and is much more laborious than most colorimetric determinations in current use. Scattered reports have appeared applying the ninhydrin colorimetric reaction to the study of plasma $\alpha$-amino nitrogen, but the methods described have been either arduous to perform (*4*), or have not been correlated with the standard gasometric technic alluded to previously (*5, 6*).

The present study was undertaken to determine whether the ninhydrin colorimetric method described by Rubinstein and Pryce (*6*) could satisfactorily replace the gasometric analysis. Initially, our results were unsatisfactory due to high blank readings, poor replicability, and grossly elevated plasma values. However, reduction of the *p*H of the ninhydrin reaction and modifications in the method for plasma protein precipitation and deproteinized plasma filtration resulted in disappearance of the shortcomings described above as well as good agreement with data from the gasometric technic. The authors believe that the modified ninhydrin colorimetric method, as reported herein, provides an accurate, simple, rapid means for the determination of plasma $\alpha$-amino nitrogen suitable for use in any clinical laboratory.

## Methods

### Reagents and Apparatus

*Buffer* Add 5 gm. of disodium ethylenediamine tetra-acetate and 240 mg. of potassium cyanide to an acetate buffer composed of 300 ml. of 2M sodium acetate (164 gm./L. anhydrous salt) and 700 ml. of 2N acetic acid (120 ml. glacial acetic acid per liter). When preserved with a few drops of chloroform this solution is stable indefinitely, and maintains a *p*H of 4.3 during color development.

*Phenol* Dissolve 400 gm. of phenol in 100 ml. of absolute ethanol by heating. After the solution has cooled, add approximately 5 gm. of Permutit.* Stir the solution well for 10 min. and filter. Slight yellow discoloration of the phenol reagent due to oxidation does not interfere with subsequent determinations.

*Ninhydrin* Dissolve 6 gm. of ninhydrin in 100 ml. of absolute ethanol by vigorous stirring. Add approximately 2 gm. of Permutit and stir the mixture for 10 min. Then filter the pale, yellow-green solution and store in a brown bottle.

*Amino acid stock standard* Prepare a 0.05 M glycine standard by dissolving 375.5 mg. of glycine in distilled water and diluting to 100

*Fisher Scientific Company, Fairlawn, N. J.

ml. The solution should be refrigerated following addition of a few drops of chloroform. (Any amino acid which gives full color development with ninhydrin should give results comparable to those with glycine, but standard curves should be prepared. See Troll and Cannon (*7*) or Rubinstein and Pryce (*6*).

*Amino acid working standard* Dilute 4 ml. of amino acid stock standard to 100 ml. with distilled water. This solution must be kept frozen when not in use to avoid bacterial contamination and resultant incomplete color yield.

*Plasma protein precipitant* Mix 1 part 13.3% (w/v) sodium tungstate with 9 parts 0.11 N hydrochloric acid. This solution is unstable and must be made fresh each time it is used.

For the color development apparatus 8 × ¾-in. test tubes are used. All glassware used in the procedure must be scrupulously cleaned to avoid contamination which may result in incomplete color development, falsely high absorbance readings, and high blank determinations.

## Procedure

### Plasma Collection and Deproteinization

Obtain heparinized venous blood samples to prevent coagulation. Within 30 min. after collection of the sample, centrifuge the blood for 10 min. at 600g and pipet the supernatant plasma into a clean test tube. Pipet 0.3 ml. of plasma into 0.7 ml. of distilled water in a conical centrifuge tube, and add 3 ml. of the tungstic acid precipitant reagent, with shaking. After centrifuging the tube for 10 min. at 600g, decant the supernatant through a glass-wool plug. For the blank, use 3 ml. of the tungstic acid reagent and 1 ml. of distilled water, and for the standard, 3 ml. of the tungstic acid reagent and 1 ml. of the working standard.

### Color Development

Pipet 0.5 ml. of the plasma supernatant, standard, or blank into 8 × ¾-in. test tubes. Add 0.5 ml. of the buffer solution and 5.0 ml. of phenol reagent to each tube, followed by vigorous shaking until clear. Then add 0.5 ml. of the ninhydrin solution and shake the solution again. Place the tubes in an actively boiling water bath for 5 min. (It is unnecessary to stopper the tubes while heating). Cool the tubes in water at room temperature for 10 min., and then shake them vigorously and equally for 30 sec. After shaking, the blank solution turns pale yellow,

and the test solutions take on the typical purple color. Allow the tubes to stand for at least 10 min. after shaking, before reading the absorbance at 570 mμ. in any standard colorimeter. The color fades slowly and proportionately for several hours. When read against water the absorbance of the blank is between 0.075 and 0.110 for a light path length of 1 cm.

### Calculation

The sample analyzed is equal to 300/8 μl. of plasma. The standard analyzed contains 3.5 μg. of alpha-amino nitrogen. Therefore, the final equation becomes:

$$\frac{(\text{O.D. test solution}) - (\text{O.D. blank})}{(\text{O.D. standard}) - (\text{O.D. blank})} \times \frac{8}{3} \times 3.5 = \text{mg. } \alpha\text{-amino N per 100 ml. plasma}$$

## Results

### Modification of Plasma Protein Precipitation Technic

Initially, the plasma proteins were precipitated with the sulfuric acid-sodium tungstate reagent described by Rubinstein and Pryce (*6*). As these investigators pointed out, the deproteinized plasma filtrate obtained with this technic always became turbid when the phenol reagent was added. The turbidity developed with standard solutions and water blanks as well as plasma samples, indicating that the clouding of the solution resulted from some interaction between the two chemical reagents, rather than from a reaction with a plasma constituent. Attempts were made to prevent the turbidity by dropwise addition of the phenol, vigorous shaking of the filtrate during phenol addition, and neutralization of the filtrate with sodium hydroxide, but with no success. However, replacement of the 0.11 N sulfuric acid by 0.11 N hydrochloric acid in the tungstic acid precipitant resulted in complete clearing of the solutions.

This change in technic resulted in significantly lower water blank readings (from 0.280-0.300 with the sulfuric acid to 0.175-0.200 with the hydrochloric acid) as well as marked improvement in replicability between duplicate analyses of the same sample (from 4.3% with the sulfuric acid to 0.8% with the hydrochloric acid). Of greatest importance, however, was the finding that plasma α-amino nitrogen concentrations obtained with the hydrochloric acid-sodium tungstate precipitant were 10–30% lower than those obtained with the sulfuric acid-sodium tungstate reagent. Representative data from an intravenous

**Table 1.** COMPARISON OF PLASMA α-AMINO NITROGEN VALUES OBTAINED WITH SULFURIC ACID-TUNGSTATE VERSUS HYDROCHLORIC ACID-TUNGSTATE REAGENTS

| | *Plasma α $NH_2$-N (mg./100 ml.)* | | |
|---|---|---|---|
| *Sample** | *$H_2SO_4$-tungstate* | *HCl-tungstate* | *% reduction* |
| 1 | 5.60 | 5.04 | 10 |
| 2 | 14.66 | 11.82 | 19 |
| 3 | 11.92 | 9.80 | 18 |
| 4 | 10.00 | 7.25 | 27 |
| 5 | 8.42 | 6.16 | 27 |
| 6 | 6.87 | 4.90 | 29 |
| 7 | 6.59 | 4.15 | 37 |
| 8 | 5.67 | 3.87 | 32 |
| 9 | 6.08 | 5.44 | 10 |
| 10 | 6.49 | 4.50 | 31 |
| | | | $\bar{x} = 24.0\%$ |

*Obtained before and during a 180-min intravenous amino acid tolerance study.

amino acid tolerance study are shown in Table 1, in which are given values for samples analyzed by both methods.

### Filtration with Glass Wool Versus Cotton Wool

It was necessary to filter the deproteinized plasma to remove small particles of protein which did not spin down during centrifugation. When cotton wool plugs were used, as recommended by Rubinstein and Pryce, water blank absorbance readings were very high (0.180-0.390). However, use of glass wool plugs resulted in reduction of blank readings, to values between 0.075 and 0.110. This change in procedure was also associated with a consistent 5–10% reduction in plasma α-amino nitrogen concentration, indicating that commercial cotton wool preparations contain ninhydrin-positive compounds which seriously impair their usefulness in the present determination.

### Reduction of Reaction *p*H

Several investigators (*7–9*) have reported a *p*H optimum of 5.0–5.1 for photometric ninhydrin reactions, and it was originally planned to use this *p*H in the present study. However, when color development was conducted at *p*H 5.0 with a sodium acetate-acetic acid buffer, a burgundy red color was noted rather than the typical purple associated with ninhydrin reactions. The red color appeared with standard solutions and plasma samples and was independent of plasma protein precipitation. It was observed that lowering the *p*H of the buffer to 4.3

resulted in the appearance of the expected purple color as well as significant increases in absorbance at 570 $m\mu$ when compared with the solutions obtained at *p*H 5.0. Therefore, all subsequent studies were conducted with the sodium acetate-acetic acid buffer at *p*H 4.3.

### Reproducibility of the Modified Method

With use of the hydrochloric acid-sodium tungstate precipitant and the glass wool plugs discussed above, repeated observations on a single plasma sample were obtained during a 2-week period. As noted by many other workers, repeated freezing and thawing of the plasma or the plasma filtrate resulted in no change in final plasma $\alpha$-amino nitrogen values. Thirty separate determinations on this single plasma gave a mean absorbance of $0.413 \pm 0.011$, yielding a standard deviation of 2.7%. The standard deviation was derived from the following formula:

$$\text{S.D.} = \sqrt{\frac{\Sigma\,(\bar{x} - x)^2}{n - 1}}$$

in which $\Sigma$ represents sum; $\bar{x}$, mean value; $x$, single determination; and $n$, number of determinations. All plasma samples were run in duplicate and excellent reproducibility consistently observed.

### Recovery of Standard Amino Acid Solutions from Plasma

Several investigators (*6, 7, 9*) have reported that all of the $\alpha$-amino acids normally found in plasma, except trytophane, phenylalanine, and tryosine, give full color development with ninhydrin. In the present study, glycine, L-glutamine, and monosodium glutamate were chosen as standards. As shown in the lower curves of Fig. 1, color development with increasing concentrations of the three standard amino acid solutions followed Beer's law. The color development for monosodium glutamate was noted to be about 10% greater than with glycine or L-glutamate. Addition of increasing quantities of one of the three standard amino acid solutions to plasma samples prior to protein precipitation yielded data plotted in the upper curves of Fig. 1. The stoichiometry of the ninhydrin reaction was unchanged when plasma was used, as evidenced by the equality of the slope for each amino acid in the presence or absence of plasma. Recovery of added standard amino acid ranged from 94 to 102%, and extrapolation of the curves to the ordinate yielded baseline plasma values which agreed with those actually determined analytically to within 3.0%. Glycine was arbitrari-

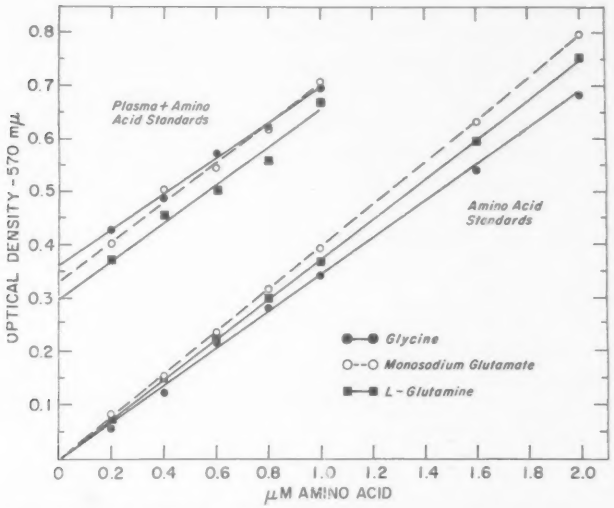


**Fig. 1.** Preparation of standard amino acid curves and recovery of added amino acids from plasma.

ly chosen as the permanent standard in the study, but any amino acid developing full color would have been satisfactory.

### Comparison with Gasometric Ninhydrin Method

Eleven fasting plasma samples from normal subjects were obtained, and α-amino nitrogen concentrations determined using both the modified ninhydrin colorimetric method described herein, and the ninhydrin gasometric technic of Hamilton and Van Slyke (*11*). As shown in Table 2, individual plasma values with the two methods agreed quite well, and the mean values obtained were virtually identical. The mean difference between results obtained colorimetrically and those determined gasometrically was only +0.09%, but the standard deviation of this difference indicated that a single sample determined by both methods could be expected to vary by ±8.8%.

### Normal Plasma Values

Fasting venous samples from 14 normal male subjects and 7 normal females have been analyzed with the present method. The over-all mean of 3.85 ± 0.44 mg.%* agrees very well with results from com-

*Value as given is mean ± 1 S.D.

**Table 2.** FASTING PLASMA α-AMINO NITROGEN DETERMINATIONS USING MODIFIED NINHYDRIN COLORIMETRIC METHOD AND NINHYDRIN GASOMETRIC TECHNIC

| *Patient* | *Sex* | *Plasma α-$NH_2$-N (mg./100 ml.)* | | *Difference from gasometric (%)* |
|---|---|---|---|---|
| | | *Colorimetric* | *Gasometric* | |
| V. H. | M | 4.28 | 5.03 | −14.9 |
| P. R. | M | 4.47 | 4.21 | + 6.2 |
| L. W. | M | 4.29 | 4.86 | −11.7 |
| S. P. | F | 4.29 | 4.32 | − 0.7 |
| P. G. | F | 4.19 | 3.78 | +10.8 |
| J. H. | M | 4.43 | 4.27 | + 3.7 |
| P. B. | M | 4.58 | 4.22 | + 8.5 |
| R. R. | F | 3.50 | 3.79 | − 7.6 |
| M. L. | F | 3.45 | 3.42 | + 0.9 |
| L. Z. | M | 3.92 | 3.55 | +10.4 |
| M. W. | F | 3.96 | 4.15 | − 4.6 |
| | | $\bar{x} \pm$ S.D. = 4.12 ± 0.36* | $\bar{x} \pm$ S.D. = 4.15 ± 0.48* | $\bar{x} \pm$ S.D. = + 0.09 ± 8.8* |

*Signifies mean ± 1 S.D.

parable previous studies: Hamilton and Van Slyke, 4.1 mg.% (*3*); Rosenblum *et al.*, 3.9 mg.% (*5*). No sex differences were observed in the present study.

## Discussion

The purple color formed during the reaction between α-amino acids and ninhydrin in acid solution has been attributed to the anion of diketohydrindylidenediketohydrindamine (DYDA) (*10–12*). Moore and Stein (*8*) developed the reaction into a convenient tool for the photometric measurement of free amino acids. Subsequently, Troll and Cannon (*7*) found that the stoichiometry of the reaction could be markedly improved by using organic rather than aqueous solvents to hasten the purple color development. More recent modifications of the method (*4, 6, 9, 13*) have been directed toward elimination of trace metal interferences, stabilization of *p*H during the reaction, and reduction in water blank determinations.

Application of the photometric ninhydrin reaction to the determination of plasma α-amino nitrogen requires suitable methods for plasma protein removal. Several reports (*3, 4, 6*) have indicated that ethanol, acetone, trichloracetic acid, colloidal iron, zinc hydroxide, cadmium hydroxide, sulfosalicylic acid, and p-toluene-sulfonic acid are unsatisfactory plasma protein precipitants when used with ninhydrin. Picric acid is satisfactory (*14*), but its use requires column chromatography

of the plasma filtrate to remove the excess picric acid, which imparts a strong yellow color to the filtrate. The hydrochloric acid-sodium tungstate precipitant used in this study is an effective protein precipitant, is easy to prepare, and does not interfere with subsequent color development. The glass wool plugs efficiently remove any particulate protein which fails to centrifuge down, thus obviating the need for special methods of deproteinized plasma handling prior to reaction with ninhydrin.

Rosenblum *et al.* (5) have shown that colorimetric estimations of plasma α-amino nitrogen in uremic subjects must be corrected for the increased blood urea nitrogen present. In the present report, the good agreement with the very specific gasometric ninhydrin technic indicates that although urea, ammonia, and some amines will react with ninhydrin to give a purple color, their presence in normal plasma provides a negligible source of error.

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# Protein Electrophoretic Analyses of Serum of Cancer Patients

*W. Donald Graham*

**The mean relative proportions of a number of blood serum protein fractions assessed electrophoretically are altered in active cancer. The changes in number and degree are generally greater than in other diseases; this may be a reflection of the seriousness of the condition and the extent of tissue involvement. A relative lowering of the proportion of albumin and an increase in that of the various globulins in "active" cancer was absent or much less apparent in treated cases.**

**Significant changes differ according to location of the malignancy. Proportions of the $\beta$-globulins were not elevated in cancer of the gastrointestinal tract nor were those of the $\gamma$-globulins in prostatic cancer. Within the $\alpha_2$-globulins the central subfraction proportion was elevated most where tissue involvement was greatest, although in breast or prostate cancer the most pronounced alteration occurred in the proportion of the first $\alpha_2$-globulin subfraction. The subfraction of $\gamma$-globulin increased in proportion in cancer of the gastrointestinal tract differed from that elevated in cancer of the lung. Further refinement in fractionation techniques may reveal major differences in the serum protein distribution in different neoplasms. A considerable elevation of the $\alpha_2$-globulin proportion above the normal may be regarded as a sign of a poor prognosis.**

**Age had little significant effect with the exception of a rise in the third $\beta$-globulin subfraction proportion, which went through a maximum in the 50-59 year age group. The relative concentration of this same fraction was elevated in cardiovascular disease in hypertension and hypercholesterolemia.**

A GREAT MANY STUDIES of the blood serum proteins of cancer patients have been made using paper electrophoretic separations in an effort to find some change that might be useful in the diagnosis or evaluation of treatment of the disease (*1–5*). In general these studies have indi-

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cated that in fairly well-advanced cancer there is a decrease in the proportion of albumin and increases in the proportions of $\alpha_2$- and frequently the $\gamma$-globulins of the serum proteins. It is evident that these rather nonspecific changes are not of very great diagnostic value since they occur too late in the course of the disease.

Analytical data on a few cancer cases were obtained using a very simply applied paper electrophoretic procedure which, through the inclusion of nonionic surfactants in the buffer, permitted more extensive fractionation of the serum proteins (*5*). Since this increased detail might prove to be useful in the diagnosis of cancer, it was considered that the situation should be re-examined.

## Method and Material

Sera from 253 cancer patients, from 273 patients free of organic disease, and from 785 others, ambulatory but suffering from a wide variety of non-neoplastic diseases, were analyzed electrophoretically using the procedure described by Graham (*5*). Serum from many of these patients was analyzed on more than one occasion. Analytical results pooled by groups according to disease are presented for 994 patients in Table 1. It should be noted that the data on individual protein fractions throughout this paper are reported as percentages of the total serum protein and not in terms of absolute amounts. Conversion of the data into absolute amounts was not carried out because highly significant deviations from normal protein levels were not observed, such translation from proportions to absolute amounts has various pitfalls, and it is considered that a change in protein fraction proportion is informative regardless of whether total protein is increased or decreased in absolute amount.

Some 480 analyses were not included in Table 1 because (1) the patient had more than one disease, (2) the numbers with any specific disease were not sufficiently large, or (3) the results were from repeated analyses done at various times on the same patient. The results on cancer patients were divided into two categories—those from patients with cancer known to be active at the time of sampling and those from patients treated at least one year previous to sampling and in whom no active recurrence had been noted. In this group were undoubtedly some in whom the disease was active but undetected.

In all instances the standard deviations of the means were calculated and the significance of the deviations from the normal mean value was

assessed using the Student "t" test (*6*). Where P was <0.05 but >0.01 the deviation was regarded as a tendency and is marked with a dagger in the table. Where P was <0.01 but >0.001 or where P was <0.001 the differences were regarded as significant and highly significant, re-

**Table 1.** RESULTS OF ELECTROPHORETIC ANALYSES OF

| *Condition* | *No. of cases* | *Albumin (% ± S.D.*)* | *Globulins (% ± S.D.*)* | | | | |
|---|---|---|---|---|---|---|---|
| | | | $\alpha_1$ | $\alpha^1_2$ | $\alpha^2_2$ | $\alpha^2_2$ | *Total* $\alpha_2$ |
| Normal | 273 | 44.9 | 6.1 | 1.6 | 7.4 | 2.0 | 11.0 |
| | | ± 4.8 | 1.3 | 0.6 | 1.8 | 0.7 | 2.0 |
| Hypertension | 21 | 43.4 | 5.8 | 1.9† | 7.4 | 2.1 | 11.4 |
| | | ± 2.2 | 2.2 | 0.7 | 1.4 | 0.8 | 2.2 |
| Cardiovascular | 75 | 43.6† | 6.1 | 1.7 | 8.0‡ | 2.1 | 11.8§ |
| | | ± 4.5 | 1.5 | 0.6 | 1.9 | 0.7 | 1.9 |
| Hypercholesterolemia | 28 | 43.0† | 6.0 | 1.6 | 7.3 | 2.0 | 10.8 |
| | | ± 4.6 | 1.5 | 0.5 | 1.4 | 0.8 | 1.8 |
| Obesity | 35 | 44.3 | 6.4 | 1.8 | 7.5 | 2.4 | 11.6 |
| | | ± 4.3 | 1.7 | 0.7 | 1.3 | 0.8 | 1.8 |
| Ulcer | 28 | 44.5 | 6.1 | 1.9† | 7.3 | 2.2 | 11.4 |
| | | ± 3.6 | 1.4 | 0.9 | 1.4 | 0.7 | 1.9 |
| Nervous anxiety | 57 | 44.3 | 6.2 | 1.7 | 7.6 | 2.2 | 11.5 |
| | | ± 3.8 | 1.5 | 0.6 | 1.6 | 0.8 | 2.0 |
| Chronic infection | 73 | 44.6 | 6.1 | 1.7 | 7.4 | 1.9 | 11.0 |
| | | ± 4.2 | 1.4 | 0.7 | 1.7 | 0.7 | 2.0 |
| Acute infection | 15 | 45.2 | 5.6 | 1.6 | 8.0 | 2.0 | 11.6 |
| | | ± 5.3 | 1.2 | 0.5 | 2.2 | 0.9 | 2.2 |
| Arthritis | 52 | 42.7† | 6.3 | 1.7 | 8.1† | 2.0 | 11.8† |
| | | ± 4.6 | 1.3 | 0.5 | 1.8 | 0.8 | 1.9 |
| Diabetes | 27 | 45.4 | 6.2 | 1.8 | 7.4 | 1.8 | 11.0 |
| | | ± 5.8 | 1.1 | 0.7 | 1.4 | 0.5 | 2.1 |
| Hyperthyroid | 10 | 46.1 | 5.6 | 1.5 | 7.5 | 1.4‡ | 10.4 |
| | | ± 4.2 | 0.8 | 0.4 | 1.8 | 0.4 | 1.8 |
| Hypothyroid | 16 | 44.6 | 6.1 | 1.7 | 7.2 | 1.9 | 10.8 |
| | | ± 4.7 | 1.4 | 0.6 | 1.7 | 0.7 | 2.0 |
| Gall bladder | 21 | 44.7 | 5.8 | 1.9† | 7.4 | 1.9 | 11.4 |
| | | ± 4.5 | 0.8 | 0.7 | 0.2 | 0.8 | 2.4 |
| Anemia | 10 | 41.1† | 6.7 | 1.7 | 7.4 | 2.2 | 11.2 |
| | | ± 1.7 | 1.5 | 0.7 | 2.4 | 0.8 | 2.3 |
| Active cancer | 156 | 39.5§ | 6.4‡ | 2.0§ | 8.8§ | 2.2 | 13.0§ |
| | | ± 4.9 | 1.5 | 0.8 | 2.4 | 0.9 | 2.7 |
| Inactive cancer | 97 | 45.3‡ | 6.4† | 1.7 | 7.8 | 2.0 | 11.5 |
| | | ± 3.7 | 1.3 | 0.6 | 1.8 | 0.6 | 2.0 |

*Lower figure of each pair.
†P (significance of differences from normal means) <0.05.
‡P <0.01.
§P <0.001.

spectively. It must be realized that, for individual serum analyses, these differences must be considerably larger before they will assume significance.

Where the patient had more than one disease entity the results were

BLOOD SERA FROM NORMAL AND DISEASED PERSONS

| *Globulins (% ± S.D.*)* | | | | | | | | |
|---|---|---|---|---|---|---|---|---|
| $\beta_1$ | $\beta_2$ | $\beta_3$ | *Total* $\beta$ | $\gamma_1$ | $\gamma_2$ | $\gamma_3$ | $\gamma_4$ | *Total* $\gamma$ |
| 8.5 | 6.3 | 5.5 | 20.3 | 4.4 | 6.5 | 5.5 | 1.3 | 17.7 |
| 1.6 | 1.7 | 1.5 | 2.5 | 1.7 | 1.8 | 1.7 | 0.6 | 3.0 |
| 8.4 | 6.8 | 6.2† | 21.4† | 4.7 | 7.0 | 4.7† | 1.3 | 17.7 |
| 1.4 | 1.8 | 1.6 | 2.4 | 1.1 | 2.4 | 2.1 | 0.5 | 2.4 |
| 8.5 | 6.6 | 6.2§ | 21.3‡ | 4.4 | 6.2 | 5.3 | 1.3 | 17.2 |
| 1.9 | 1.7 | 2.0 | 2.4 | 1.4 | 1.7 | 1.7 | 0.7 | 3.4 |
| 9.2† | 6.7 | 6.1† | 22.0§ | 4.9 | 6.6 | 5.4 | 1.1 | 18.0 |
| 2.1 | 1.7 | 1.9 | 2.8 | 1.4 | 1.7 | 1.2 | 0.3 | 3.2 |
| 8.7 | 6.3 | 5.4 | 20.4 | 4.1 | 6.8 | 5.4 | 1.0 | 17.3 |
| 1.6 | 1.5 | 1.3 | 2.2 | 1.3 | 2.0 | 2.8 | 0.4 | 3.2 |
| 8.3 | 6.1 | 5.6 | 20.0 | 4.7 | 6.4 | 5.3 | 1.6† | 18.0 |
| 1.3 | 1.9 | 1.7 | 2.3 | 2.3 | 1.6 | 1.9 | 0.9 | 3.0 |
| 8.3 | 6.0 | 5.9 | 20.2 | 4.2 | 6.8 | 5.4 | 1.3 | 17.7 |
| 1.6 | 1.7 | 1.5 | 2.8 | 1.3 | 2.1 | 2.0 | 0.5 | 3.5 |
| 8.3 | 6.2 | 5.6 | 20.1 | 4.5 | 7.1† | 5.2 | 1.2 | 18.0 |
| 1.3 | 1.7 | 1.6 | 2.5 | 1.2 | 2.1 | 1.7 | 0.6 | 3.5 |
| 8.3 | 6.0 | 4.8 | 19.1 | 4.5 | 7.1† | 5.4 | 1.4 | 18.4 |
| 2.2 | 1.2 | 1.4 | 1.8 | 1.3 | 1.9 | 1.5 | 0.6 | 3.8 |
| 8.2 | 6.9† | 5.7 | 20.8 | 4.6 | 6.9 | 5.6 | 1.4 | 18.4 |
| 2.2 | 1.7 | 2.0 | 2.8 | 1.6 | 1.6 | 1.7 | 0.6 | 4.2 |
| 8.1 | 6.5 | 5.6 | 20.2 | 4.5 | 5.6† | 6.0 | 1.3 | 17.3 |
| 1.6 | 1.6 | 1.7 | 3.0 | 1.2 | 2.5 | 2.0 | 0.6 | 3.5 |
| 8.4 | 6.8 | 5.0 | 20.2 | 4.0 | 6.7 | 5.5 | 1.4 | 17.7 |
| 1.8 | 1.3 | 1.3 | 2.7 | 1.5 | 0.7 | 1.6 | 0.6 | 3.2 |
| 8.7 | 5.9 | 5.9 | 20.5 | 4.4 | 6.6 | 5.4 | 1.5 | 18.0 |
| 1.8 | 0.9 | 1.8 | 2.7 | 1.4 | 1.5 | 1.8 | 0.6 | 2.9 |
| 8.6 | 6.1 | 5.4 | 20.1 | 3.8 | 7.3 | 5.6 | 1.4 | 18.1 |
| 1.2 | 2.2 | 1.2 | 2.0 | 1.5 | 2.7 | 2.1 | 0.9 | 4.3 |
| 9.5† | 6.1 | 5.1 | 20.7 | 4.4 | 7.2 | 7.4§ | 1.3 | 20.3‡ |
| 2.4 | 2.0 | 1.7 | 3.7 | 1.6 | 1.9 | 2.8 | 0.3 | 4.6 |
| 8.2 | 7.4§ | 5.9† | 21.6§ | 4.4 | 7.5§ | 6.2§ | 1.4‡ | 19.5§ |
| 1.7 | 1.7 | 1.7 | 2.8 | 1.5 | 2.6 | 2.2 | 0.7 | 4.3 |
| 8.4 | 6.7† | 5.5 | 20.6 | 4.8† | 6.6 | 5.5 | 1.3 | 18.2 |
| 1.6 | 1.4 | 1.5 | 2.7 | 1.4 | 2.0 | 2.0 | 0.5 | 3.5 |

virtually impossible to interpret. In general the means for the analytical data did not differ significantly from those obtained where the patients had only one specific disease. But in several instances, notably in diabetes, the picture in the presence of complicating diseases was different from that in the uncomplicated disease. To avoid any confusion, data from patients with more than one diagnosed disease were eliminated from consideration in the presentation that follows.

## Results and Discussion

The normal mean values listed in Table 1 are in quite close agreement with those reported earlier on 70 cases and appear to be valid for this particular procedure as applied in this laboratory.

In various non-neoplastic diseases studied, the changes from the normal were usually of a low order of significance. A tendency toward lowered albumin percentage was noted in vascular disease, arthritis, hypercholesterolemia, and the anemias. The $\alpha_1$-globulin proportion was not altered in any disease state investigated other than cancer, although it would appear that a significant increase might have been found in the anemias if sufficient cases had been examined. The $\alpha_2$-globulins proportions were elevated in vascular disease and, as in active cancer, the main change was in the second subfraction. In arthritis there was a tendency toward elevation of the proportions of these same fractions. In hypertension, ulcers of the gastrointestinal tract, and gall bladder diseases, there was a tendency toward elevation of the first $\alpha_2$ subfraction proportion. In hyperthyroidism there appeared to be a significant decrease in the relative percentage of the third $\alpha_2$ subfraction.

In the $\beta$-globulins there was a tendency toward an increased proportion in hypertension, with the main effect localized in the third subfraction. A similar but much more profound alteration occurred in vascular disease. There was a rather similar finding in hypercholesterolemia where most of the change was localized in the first and third subfractions. These results will be considered further in another context. Minor tendencies toward change in the second $\beta$-globulin subfraction occurred in arthritis (increase) and in the first $\beta$-subfraction in anemia (increase).

In the $\gamma$-globulins, among non-neoplastic diseases, significant increases in their proportions occurred only in the anemias, with the main change in the third subfraction. The significance of this finding

is not apparent. In several other diseases there were tendencies toward elevation or depression of the relative amounts of particular subfractions but none was of great statistical significance.

In the 97 cancer patients without obvious signs of activity of the disease the deviations of serum protein fractions from normal distribution were limited. There was a significant decrease in the proportion of albumin and a tendency toward elevations in those of the $\alpha_1$-globulins, the second $\beta$-globulin subfraction, and the first $\gamma$-globulin subfraction. These changes are considered to be reflections of the probable presence in this group of a number of patients in whom an exacerbation of the disease was occurring.

In the sera of cancer patients with active disease there were highly significant deviations from normality. Albumin proportions were decreased with resulting increases in all other gross fractions, expressed as percentages of the total protein. The increase in proportion of $\alpha_2$-globulins could be traced to highly significant increases in those of the first and second subfractions. In the $\beta$-globulins virtually all the proportional increase was localized in the second subfraction, although the third subfraction also showed a tendency toward elevation. In the $\gamma$-globulins, significant increases occurred in the relative percentage of all subfractions but the first. These findings are in remarkably close agreement with those found previously (*5*), considering the very limited number of cases reported on at that time.

In some instances numbers made it possible to extract subgroups from the analysis for the active cancer group. These are listed in Table 2 together with the means for the analytical data and estimates of the significance of deviations from the normal.

Some interesting observations can be made. In all but skin cancer there was a highly significant depression of the albumin proportion. This is considered to be a reflection of the general debility of the cancer patient with an appreciable amount of invasive growth. Only in cancer of the gastrointestinal tract was there a highly significant elevation of the $\alpha_1$-globulin proportion. This, again, may be a reflection of the amount of tissue involvement.

The total $\alpha_2$-globulin proportions were significantly elevated in all but skin cancer. In most instances this was due to an elevation of the main $\alpha_2$-component although in cancer of the breast and prostatic cancer the elevation occurred more in the satellite subfractions. It would be interesting to determine the area of migration of acid phosphatase

(which is known to migrate in the general $\alpha_2$ zone) in these latter instances. In cancer of the gastrointestinal tract, the apparent elevation of the first subfraction proportion may have been caused by a poor separation of the overshadowing increase in the central subfraction.

Total $\beta$-globulin was increased in proportion primarily in cancer of the breast or prostate and in the miscellaneous group. In all of these the increase was almost entirely in the second subfraction. A striking observation here was the lack of change in the proportion of the $\beta$-globulins in those suffering from cancer of the gastrointestinal tract while, in skin cancer, the second $\beta$-globulin subfraction was the only one showing involvement. The disproportionate increase in one particular subfraction in many of these cases may account for the frequently reported split in the $\beta$-globulin peak found by the ordinary paper electrophoresis procedures (*7*).

In the $\gamma$-globulin area the effects were scattered; perhaps the most surprising was the lack of deviation from normality of the $\gamma$-globulin distribution of the prostatic cancer patient sera.

Explanations of why these variations in serum protein distribution occur in the several categories of cancer studied will have to await further investigation. It is encouraging that differences have been

**Table 2.** RESULTS OF ELECTROPHORETIC ANALYSES OF

| *Cancer* | *No. of cases* | *Albumin (% ± S.D.*)* | *Globulins (% ± S.D.*)* | | | | |
|---|---|---|---|---|---|---|---|
| | | | $\alpha_1$ | $\alpha'_2$ | $\alpha''_2$ | $\alpha^3_2$ | *Total* $\alpha_2$ |
| All cases | 156 | 39.5§ | 6.4‡ | 2.0§ | 8.8§ | 2.2 | 13.0§ |
| | | ± 4.9 | 1.5 | 0.8 | 2.4 | 0.9 | 2.7 |
| Skin | 14 | 43.1 | 5.9 | 2.1 | 6.9 | 2.1 | 11.1 |
| | | ± 4.2 | 1.3 | 0.7 | 1.3 | 0.6 | 1.7 |
| Gastrointestinal | 21 | 37.9§ | 7.3§ | 2.1§ | 9.3§ | 2.5† | 13.9§ |
| | | ± 5.5 | 0.7 | 0.8 | 2.7 | 1.6 | 3.6 |
| Lung | 19 | 37.0§ | 6.9† | 1.8 | 9.7§ | 2.0 | 13.5§ |
| | | ± 6.0 | 1.5 | 0.8 | 2.7 | 0.8 | 2.8 |
| Breast | 25 | 39.7§ | 6.5 | 2.1§ | 7.9 | 2.4† | 12.4‡ |
| | | ± 3.3 | 1.5 | 0.6 | 1.6 | 0.7 | 1.7 |
| Prostate | 14 | 40.5§ | 6.0 | 2.3§ | 8.4 | 2.4† | 13.0§ |
| | | ± 4.9 | 1.1 | 0.9 | 1.8 | 0.8 | 2.1 |
| Misc. | 63 | 39.6§ | 6.1 | 1.8 | 9.3§ | 2.1 | 13.2§ |
| | | ± 4.1 | 1.6 | 0.7 | 2.5 | 0.8 | 2.8 |

*Lower figure of each pair.
†P (significance of differences from normal means) $<0.05$.
‡P $<0.01$.
§P $<0.001$.

demonstrated. Most of the literature reports hold that the over-all picture represents one of widespread tissue involvement and "probably does not represent a specific abnormality due to the presence of malignancy" (*8*). The present findings are generally in accord with this statement. They do suggest, however, that the changes are not as nonspecific as has been assumed; that variations in the number, kind, and extent of the changes may be related to the particular malignancy; that refinement of the analytical procedure has brought out points of difference; and that further refinement holds promise of increasing the knowledge to be gained by this approach.

On a number of cancer patients, analyses were performed on more than one occasion with the aim of establishing the value of this procedure in assessing the effectiveness of a treatment or in establishing prognosis. Rarely were sufficient analyses performed to establish a clear picture, with the one notable exception, described in the following case report.

The patient, a white male aged 54, appeared Dec. 5, 1959, with a large retroperitoneal mass considered metastatic from a left testicular tumor of some 15 year's duration. At this time the electrophoretic analysis was as shown in Table 3.

The primary seminoma was excised Dec. 7, 1959, and the patient was given postopera-

BLOOD SERA FROM PATIENTS WITH ACTIVE CANCER

| *Globulins (% ± S.D.*)* | | | | | | | | |
|---|---|---|---|---|---|---|---|---|
| $\beta_1$ | $\beta_2$ | $\beta_3$ | *Total* $\beta$ | $\gamma_1$ | $\gamma_2$ | $\gamma_3$ | $\gamma_4$ | *Total* $\gamma$ |
| 8.2 | 7.4§ | 5.9† | 21.6§ | 4.4 | 7.5§ | 6.2§ | 1.4‡ | 19.5§ |
| 1.7 | 1.7 | 1.7 | 2.8 | 1.5 | 2.6 | 2.2 | 0.7 | 4.3 |
| 8.3 | 7.6‡ | 5.6 | 21.5 | 4.1 | 7.3 | 5.6 | 1.4 | 18.3 |
| 1.6 | 2.4 | 0.8 | 2.3 | 1.1 | 1.9 | 1.7 | 0.5 | 3.0 |
| 8.2 | 6.7 | 5.6 | 20.6 | 5.2† | 7.4† | 6.1 | 1.7‡ | 20.4§ |
| 2.4 | 1.8 | 2.1 | 2.9 | 1.7 | 2.7 | 1.3 | 0.2 | 4.6 |
| 7.9 | 7.5‡ | 6.4† | 21.8† | 4.5 | 7.8‡ | 7.0§ | 1.4 | 20.7§ |
| 1.4 | 1.4 | 1.4 | 2.9 | 1.3 | 2.6 | 1.9 | 0.7 | 4.8 |
| 8.8 | 7.2† | 6.0 | 22.0‡ | 4.2 | 7.2 | 6.5† | 1.5 | 19.4† |
| 1.4 | 1.4 | 1.7 | 2.4 | 1.2 | 1.8 | 2.1 | 0.6 | 3.0 |
| 8.7 | 8.7§ | 5.8 | 23.2§ | 3.8 | 7.1 | 4.9 | 1.4 | 17.2 |
| 1.6 | 1.6 | 1.5 | 2.0 | 0.9 | 4.4 | 2.3 | 0.7 | 6.1 |
| 8.0† | 7.4§ | 6.0† | 21.4‡ | 4.3 | 7.6§ | 6.2† | 1.4 | 19.5§ |
| 1.7 | 1.6 | 1.7 | 3.0 | 1.6 | 2.5 | 2.5 | 0.8 | 4.6 |

**Table 3.** CHANGES IN ELECTROPHORETIC SERUM PROTEIN

| *Analysis No.* | *Date* | *Albumin (%)* | *Globulins (%)* | | | | |
|---|---|---|---|---|---|---|---|
| | | | $\alpha_1$ | $\alpha'_2$ | $\alpha^2_2$ | $\alpha^3_2$ | *Total* $\alpha_2$ |
| 1 | 12/ 5/59 | 30.2 | 9.6 | 1.1 | 7.4 | 1.5 | 10.0 |
| 2 | 2/ 9/60 | 38.0 | 7.0 | 1.8 | 6.5 | 1.0 | 9.3 |
| 3 | 2/29/60 | 41.6 | 7.0 | 1.8 | 6.0 | 1.0 | 8.8 |
| 4 | 5/31/60 | 37.4 | 6.3 | 0.8 | 10.7 | 1.9 | 13.4 |

tive $Co^{60}$ radiation therapy which led to virtually complete regression of the abdominal tumor mass. On Feb. 9, 1960, analysis (Table 3) showed a marked increase in the albumin proportion and concomitant decreases in the proportions of the $\gamma$-globulins. During the next few weeks the patient received $Co^{60}$ radiation to the mediastinal nodes. Clinically, the patient appeared to be much improved when seen Feb. 29, 1960. The blood protein picture on this date (Table 3) also showed a continued improvement. The relative percentage of albumin had returned within normal limits and that of the $\alpha_2$-globulin had decreased slightly. The proportion of $\gamma$-globulins remained fairly high. During these weeks the $\beta$-globulins showed fluctuations of unknown significance, in part perhaps reflecting the postprandial state of the patient at the time of sampling.

**Table 4.** EFFECT OF AGE ON ELECTROPHORETIC

| *Age* | *Number of patients* | *Albumin (% ± S.D.*)* | *Globulins (% ± S.D.*)* | | | | |
|---|---|---|---|---|---|---|---|
| | | | $\alpha_1$ | $\alpha'_2$ | $\alpha^2_2$ | $\alpha^3_2$ | *Total* $\alpha_2$ |
| 6-19 | 11 | 45.8 | 5.6 | 1.6 | 7.8 | 1.7 | 11.1 |
| | | ± 3.1 | 1.1 | 0.4 | 1.8 | 0.7 | 1.9 |
| 20-29 | 24 | 45.7 | 5.6 | 1.6 | 7.7 | 1.8 | 11.1 |
| | | ± 3.4 | 1.2 | 0.6 | 1.8 | 0.4 | 1.8 |
| 30-39 | 49 | 46.0 | 5.8 | 1.7 | 7.2 | 1.9 | 10.8 |
| | | ± 4.9 | 0.9 | 0.1 | 1.9 | 0.6 | 1.9 |
| 40-49 | 59 | 45.0 | 6.3 | 1.7 | 7.3 | 1.9 | 10.9 |
| | | ± 4.1 | 1.1 | 0.6 | 1.7 | 0.7 | 1.9 |
| 50-59 | 70 | 44.3 | 6.1 | 1.5 | 7.3 | 2.1 | 10.9 |
| | | ± 6.5 | 1.5 | 0.5 | 1.7 | 0.8 | 2.0 |
| 60-69 | 42 | 43.9 | 6.1 | 1.6 | 7.7 | 2.0 | 11.3 |
| | | ± 3.5 | 1.5 | 0.6 | 1.6 | 0.7 | 3.4 |
| 70-86 | 18 | 43.6 | 6.1 | 1.6 | 7.7 | 2.2 | 11.5 |
| | | ± 3.5 | 1.5 | 0.6 | 1.9 | 0.7 | 2.0 |
| All cases | 273 | 44.9 | 6.1 | 1.6 | 7.4 | 2.0 | 11.0 |
| | | ± 4.8 | 1.3 | 0.6 | 1.8 | 0.7 | 2.0 |

*Lower figure of each pair.
†P (significance of differences from the over-all normal means) $<0.05$.
‡P $<0.01$.

PATTERN OF A CANCER PATIENT DURING TREATMENT

| *Globulins (%)* | | | | | | | | |
|---|---|---|---|---|---|---|---|---|
| $\beta_1$ | $\beta_2$ | $\beta_3$ | *Total* $\beta$ | $\gamma_1$ | $\gamma_2$ | $\gamma_3$ | $\gamma_4$ | *Total* $\gamma$ |
| 8.4 | 6.3 | 4.4 | 19.1 | 9.4 | 11.2 | 9.1 | 1.4 | 31.1 |
| 5.6 | 8.7 | 6.8 | 21.1 | 7.6 | 8.7 | 7.0 | 1.3 | 24.6 |
| 7.0 | 5.2 | 5.6 | 17.8 | 5.6 | 10.5 | 7.5 | 1.2 | 24.8 |
| 5.8 | 4.7 | 4.4 | 14.9 | 5.0 | 5.5 | 13.7 | 3.8 | 28.0 |

The last sample was obtained May 31, 1960, at which time a recurrence of the abdominal invasion was noted. A reversion to the cancer type of blood serum protein distribution had occurred (Table 3). The clinical course continued downhill and the patient died July 1, 1960.

Wildhack (*9*) found that elevation of the $\alpha_2$-globulin above 13% of the total serum protein indicated a very poor prognosis in Hodgkins' disease. A consideration of data obtained in this laboratory would suggest that this conclusion is generally applicable in cancer—an elevated serum $\alpha_2$-globulin proportion is a sign of poor prognosis, with

ANALYSES OF NORMAL BLOOD SERA

| *Globulins (% ± S.D.*)* | | | | | | | | |
|---|---|---|---|---|---|---|---|---|
| $\beta_1$ | $\beta_2$ | $\beta_3$ | *Total* $\beta$ | $\gamma_1$ | $\gamma_2$ | $\gamma_3$ | $\gamma_4$ | *Total* $\gamma$ |
| 9.0 | 5.5 | 4.4† | 18.9 | 4.2 | 6.6 | 6.6 | 1.0 | 18.5 |
| 1.8 | 1.2 | 1.4 | 2.2 | 0.8 | 1.8 | 2.4 | 0.2 | 2.8 |
| 8.5 | 5.9 | 4.9 | 19.3 | 4.5 | 6.7 | 5.6 | 1.3 | 18.1 |
| 1.2 | 1.6 | 1.8 | 2.2 | 1.3 | 1.7 | 1.5 | 0.6 | 2.8 |
| 8.2 | 6.1 | 5.1 | 19.4† | 4.2 | 6.7 | 5.8 | 1.3 | 18.0 |
| 1.6 | 1.4 | 1.3 | 2.3 | 1.4 | 2.4 | 1.8 | 0.5 | 3.5 |
| 8.5 | 6.4 | 5.3 | 20.2 | 4.5 | 6.6 | 5.2 | 1.2 | 17.5 |
| 1.2 | 1.7 | 1.5 | 2.9 | 1.1 | 1.1 | 1.5 | 0.6 | 3.1 |
| 8.6 | 6.1 | 6.2‡ | 20.9 | 4 1 | 6.3 | 5.3 | 1.4 | 17.1 |
| 1.7 | 2.0 | 1.4 | 2.3 | 1.2 | 1.7 | 1.7 | 0.6 | 2.8 |
| 8.5 | 6.6 | 5.8 | 20.9 | 4.6 | 6.6 | 5.3 | 1.3 | 17.8 |
| 1.5 | 1.4 | 1.3 | 2.3 | 1.4 | 1.7 | 1.6 | 0.4 | 2.8 |
| 8.5 | 7.0 | 5.5 | 21.0 | 4.8 | 6.4 | 5.4 | 1.2 | 17.8 |
| 1.1 | 1.3 | 1.0 | 2.0 | 1.4 | 1.9 | 1.4 | 0.1 | 1.0 |
| 8.5 | 6.3 | 5.5 | 20.3 | 4.4 | 6.5 | 5.5 | 1.3 | 17.7 |
| 1.6 | 1.7 | 1.5 | 2.5 | 1.7 | 1.8 | 1.7 | 0.6 | 3.0 |

the severity of the disease generally correlated with the degree of elevation.

With the thought that age might be a factor in establishing the serum protein distribution (it is well-known that deviations from the adult distribution occur in young children) the data for normals were subdivided into age groups. The groupings used were 6–19, 20–29, 30–39, 40–49, 50–59, 60–69, and 70–86. The mean analytical values for serum proteins of these age groups are presented in Table 3.

In only one instance was there a significant deviation from the mean value for all ages combined and this was in the third $\beta$-globulin subfraction in the age 50–59 group. Closer inspection of these data revealed the interesting trend illustrated in Fig. 1. This particular subfraction is at a minimum proportion in the younger patients, passes through a maximum in the age 50–59 group, and then returns toward the over-all mean. Lipid staining revealed that $\beta$-lipoprotein was concentrated in this third $\beta$-globulin subfraction and probably accounted for much of the increase in percentage concentration. A similar trend has been found using ultracentrifugal analyses (*10*) and electrophoresis (*11*).

It is also interesting to note that the proportion of the third $\beta$-globulin subfraction was very significantly elevated in patients with cardiovascular disease and showed a similar tendency in hypertensive

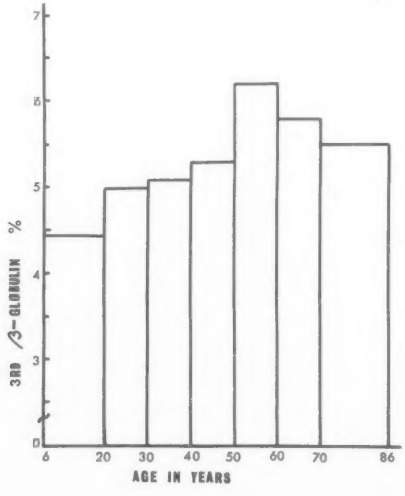


**Fig. 1.** Effect of age on the $\beta_2$-globulin proportion of normal human serum protein.

and hypercholesterolemic patients. Ages of patients with these three related disease conditions were almost entirely in the 30- to 70-year range. Combining the data from patients with these three diseases and comparing the means with the normals in the 30–70 range, both total $\beta$-globulin and the third $\beta$-globulin subfraction proportions still were significantly elevated. These findings would appear to confirm the long-suspected relation between elevated $\beta$-lipoprotein and cardiovascular disease.

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# In Vitro Stabilization of Blood Glucose with Water

*Samuel Meites and Nancy Bohman*

**The loss in glucose that shed blood undergoes on standing may be effectively inhibited for 2 hr., prior to protein precipitation, by a dilution with water of 1:80 (v/v). The inhibitory effect of water is independent of glucose concentration and temperature (up to and including room temperature). Incorporation of the dilution into the glucose oxidase method of Washko and Rice (4) makes the method particularly appropriate for the newborn.**

THERE does not seem to be any argument that glycolysis continues in the erythrocytes of shed blood (*1,3*). The rate of the glycolytic process is apparently greater in the newborn than in the adult (*2*). Glycolysis may be stopped or retarded in several ways including the immediate precipitation of blood proteins or rapid freezing; the use of preservatives such as sodium fluoride, thymol, and formaldelhyde; and prompt separation of plasma from cells with subsequent determination of plasma glucose.

The possibility that glycolysis (and the quantitatively less significant hexose phosphate shunt) may be retarded by aqueous dilution of whole blood has not been thoroughly explored in the past, though dilution is common to microprocedures. This paper presents evidence to show that dilution per se is adequate to prevent loss of glucose in the shed blood of premature infants as well as adults. Several findings incidental to the study of dilution effects are reported.

## Materials and Methods

Freshly drawn venous blood obtained from adults was used to test the effect of dilution with water on glycolysis. Throughout all experiments reported the blood glucose was analyzed immediately. Diluted

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specimens were allowed to stand for 3 hr. and the glucose value determined, along with those of heparinized whole-blood samples that served as controls, by a micro adaptation of the glucose oxidase method of Washko and Rice (*4*).

For the temperature experiment, venous blood was diluted 1:80 with water and maintained at the various temperatures indicated.

The effect of glucose concentration on the inhibition of glycolysis by dilution was studied by incorporation of appropriate volumes of a stock glucose solution into the dilution of whole blood.

*Adaptation of the ultramicro glucose oxidase method of Washko and Rice* (*4*) involved the following reagent changes:

*Phosphate buffer, 0.08 M* The concentration of phosphate buffer is doubled.

*Glucose oxidase reagent* The concentration of reagents recommended is doubled.

*Sulfuric acid, approximately 7.6 N* One volume of concentrated $H_2SO_4$ is cautiously mixed with 4 volumes of water. This reagent replaces the sulfuric acid (1:5) recommended.

### Procedure

Twenty-five microliters of whole blood (collected in a TC pipet, A.C.S. specification) is rinsed into 2 ml. of water in a 12 × 75-mm. test tube. The mixture may stand for any time up to 2 hr. provided the tube is capped. To this is added 0.1 ml. of 0.3 N $Ba(OH)_2$ and 0.1 ml. of 5% $ZnSO_4$. After mixing and centrifugation, 1.0 ml. of supernatant fluid is pipetted into a 19 × 150-mm. cuvet. A blank and standards are also run, substituting for whole blood. One milliliter of the glucose oxidase reagent is added to each cuvet and the mixture incubated for 30 min. in a water bath at 37°. Finally, 4.0 ml. of 7.6 N sulfuric acid is added to each tube. The stable pink color is measured spectrophotometrically at 540 mμ. The Coleman Junior spectrophotometer* was used exclusively in this study, but the method should be suitable for any sensitive spectrophotometer or photoelectric colorimeter.

To investigate the stabilization of glucose in the shed blood of premature infants, 16 studies of blood glucose were made on 14 premature infants. The ages of the infants varied between 1 and 72 days, and the weights between 1.16 and 2.06 kg. (Table 3). Blood was obtained by heel puncture with disposable lancets.

*Model 6-A, Coleman Instruments, Inc., Maywood, Ill.

**Table 1.** Average Percentage Loss in Glucose of Shed Whole Blood (Heparinized) at Room Temperature (24–28°)

| *Time after shedding (hr.)* | *Loss (%)* | |
|---|---|---|
| | *Adults* | *Prematures* |
| 1 | 3.0 (6) | 25.8 (11) |
| 2 | 10.4 (10) | 47.4 (13) |
| 3 | 15.8 (18) | — |

Numbers in parentheses indicate the number of subjects tested.

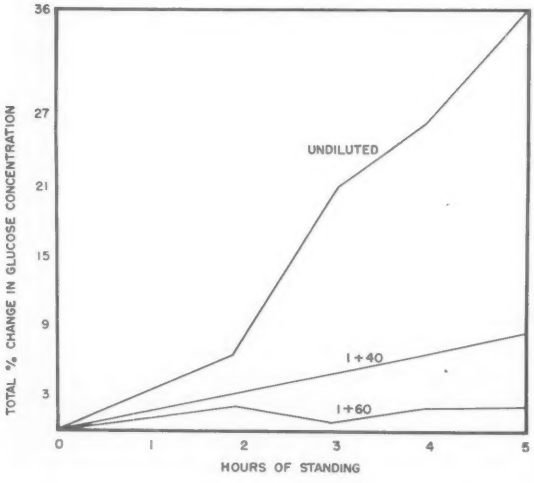


**Fig. 1.** Comparison of hourly loss of glucose in whole blood (adult, heparinized) with same blood at 1:40 and 1:60 dilution with water.

## Results

Table 1 shows the average percentage loss of glucose in shed whole blood for premature infants and adults. The rate of loss in prematures is apparently about 8 times greater during the first hour and about 4–5 times greater in 2 hr.

At 1:40 dilution (Fig. 1), change of blood glucose is within the limits of precision error (± 2.8, S.D.) for at least 2 hr., whereas at 1:60 dilution, or greater, there is no apparent loss for periods up to 5 hr.

The inhibiting effect of water appears to be independent of glucose concentration up to at least 300 mg./100 ml. Greater concentrations of glucose were not tested.

**Table 2.** Effect of Temperature on Glucose Concentration of Diluted and Undiluted Whole Blood

| *Temperature (°C)* | *Dilution (v/v)* | *% change absorbance after standing:* | |
|---|---|---|---|
| | | *2 hr.* | *3 hr.* |
| −12 | none | — | − 3.8 |
| | 1:80 | +1.1 | − 1.8 |
| − 4 | none | — | − 6.4 |
| | 1:80 | −2.9 | − 1.2 |
| 12 | none | — | −12 |
| | 1:80 | +1.1 | − 2.6 |
| 17 | none | — | −12 |
| | 1:80 | +2.3 | + 2.8 |
| 25 | none | — | −18 |
| | 1:80 | −2.0 | − 6.0 |
| 27 | none | — | −20 |
| | 1:80 | −1.6 | − 5.8 |
| 37 | none | — | −35 |
| | 1:80 | −4.7 | − 6.4 |
| 55 | none | — | −38 |
| | 1:80 | −6.7 | − 5.3 |

Table 2 demonstrates that, in whole blood maintained for 3 hr. at a series of temperatures, the glucose concentration falls regardless of the temperature. Greater fall in concentration occurs at the higher temperatures. At 1:80 dilution of whole blood with water, it is apparent that the inhibiting effect of water is independent of temperature, at least through usual room temperatures. At 37 and 55°, the inhibition may be incomplete. The 6.0 and 5.8% loss in glucose concentration at 25 and 37° after 3 hr., are exceptionally high. There is, however, no significant loss after 2 hr. of standing. It is therefore recommended that, as a rule, diluted blood should not be held longer than 2 hr. before protein precipitation is done.

Table 3 indicates the results obtained with premature infants. If the blood is allowed to stand for 2 hr. in a 1:40 dilution, glycolysis is inhibited, but not completely (Patient 1). At 1:80 dilution, the inhibition is almost complete. The greatest loss in glucose is 6.1% in one instance (Patient 6), but the remaining patients showed no significant loss.

**Table 3.** SUMMARY OF DATA ON PREMATURE INFANTS

| No. | Weight (kg.) | Age (days) | Hematocrit (%) | Initial blood sugar* (mg./100 ml.) | % change in absorbance from 0-hr. value after: 1 hr. Diluted† | 1 hr. Undil. | 2 hr. Diluted† | 2 hr. Undil. |
|---|---|---|---|---|---|---|---|---|
| 1 | 1.26 | 1 | 64 | 37 | −16.7 | −73.0 | −23.0 | −100 |
| 2 | 1.29 | 10 | — | 77 | − 2.8 | — | − 1.9 | — |
| 3 | 1.73 | 37 | — | 66 | − 1.0 | — | 0.0 | — |
| 4 | 1.97 | 72 | 31 | 71 | + 1.1 | −19.9 | — | — |
| 5 | 2.06 | 37 | 30.5 | 6[illegible] | — | — | + 1.8 | − 22.1 |
| 6 | 1.16 | 9 | 56 | 65 | − 5.1 | −34.3 | − 6.1 | − 66.5 |
| 7 | 1.19 | 11 | 42 | 91 | + 0.9 | −13.7 | − 0.9 | − 27.4 |
| 8 | 1.33 | 23 | 31.5 | 73 | + 1.3 | − 9.1 | + 2.6 | − 32.5 |
| 9 | 1.39 | 5 | 58 | 125 | + 3.8 | −12.6 | + 1.9 | − 21.4 |
| 10 | 1.45 | 23 | 41 | 83 | 0.0 | −17.6 | + 2.3 | − 30.7 |
| 11 | 1.47 | 6 | 45 | 64 | — | — | + 1.5 | − 69.0 |
| 12 | 1.68 | 4 | 76 | 68 | + 1.3 | −12.4 | + 3.8 | − 39.3 |
| 13 | 1.75 | 5 | 70 | 71 | + 1.3 | −15.8 | − 1.3 | − 46.7 |
| 14 | 1.81 | 4 | 63 | 90 | — | — | − 1.1 | − 44.4 |
| 15 | 1.91 | 1 | 39 | 37 | − 2.7 | −70.8 | 0.0 | − 96.0 |
| 16 | 1.93 | 19 | 41 | 90 | − 0.9 | − 4.1 | − 3.2 | − 20.6 |

*Blood specimens taken about 30–60 min. after feeding.
†Dilutions of blood (v/v) were 1:40 for Patients 1-5 and 1:80 for Patients 6-16.

## Discussion

The inhibiting effect of water is possibly due to dilution of enzyme and cofactor concentrations to the point where phosphorylation cannot readily occur. This dilution effect, however, may not be the only factor involved. Disruption of the erythrocyte, with subsequent dispersion of the enzymatic components, may also play a part in the inhibition, as has been demonstrated by Solomon *et al.* (*5*). This factor, on the other hand, is relatively minor, judging from our experience. Dilution of whole blood with sterile physiologic saline causes the inhibition, though not as completely as dilution with water.

At 1:80 dilution with water, the inhibiting effect is sufficient to overcome the increased glycolytic rate and the hematocrit of premature infants. This dilution was, therefore, incorporated into the glucose oxidase procedure. Although term newborns were not tested, the results should parallel those of prematures, since the rate of glycolysis is less than that of the premature (*3*).

The use of water as a diluent is ideal from the standpoint of microchemistry, as it is a convenient way to achieve blood preservation and

avoids the need for immediate protein precipitation, centrifugation, and the use of plasma, preservatives, or rapid freezing. Capillary puncture of the newborn (and certain adults as well as most children) is easier to perform than venipuncture. The use of special containers with added preservative is avoided.

While preservation of blood glucose by dilution is possible for periods up to 5 hr., this is rarely necessary. Ordinarily, blood collected at the bedside is returned to the laboratory well within 1 or 2 hr. Because the inhibiting effect of water may not persist for all blood specimens, it is advisable to precipitate the proteins at a time no longer than 2 hr. after the dilution is made.

Refrigeration or exposure of whole blood to temperatures as low as — 12° is inadequate to prevent loss of blood glucose. There is, as expected, less loss at lower than at higher temperatures. On the other hand, loss of glucose is effectively prevented by the 1:80 dilution of blood with water, and is independent of temperature within the experimental limits studied to 27°.

The basic features of the glucose oxidase method of Washko and Rice (*4*) have been retained, with the added advantages of including the 1:80 aqueous dilution of whole blood for glucose preservation and of the ultramicro quantity of blood required. The final volume of the reaction mixture may be varied to meet the specifications of any sensitive colorimetric instrument. It is only limited by the need for attaining a concentration of about 5.0 N with sulfuric acid, and the minimum volume required by the absorption cell.

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# A Quantitative Method of Determining Urinary Phenols

*Lewis A. Barness, William J. Mellman, Thomas Tedesco, Diana G. Young, and Robert Nocho*

**A quantitative method for the determination of urinary phenols is described. Data obtained in 20 children by this method are similar to averages in adults obtained by others. This is exceeded by the excretion of a phenylketonuric infant when placed on excessive intakes of phenylalanine.**

To study protein metabolism with special reference to tyrosine as well as to study the deranged metabolism of aromatic compounds in phenylketonuria and similar disorders, quantitative determination of urinary phenols is desirable. Volterra (*1*) measured three phenolic fractions in the urine by distillation. Phenols have been measured quantitatively after ethyl acetate extraction by Bray and Thorpe (*2*) and after ether extraction by Rogers *et al.* (*3*). A previous report described a method of ether extraction of urine for free, acid, and conjugated phenols (*4*). A modification of this now offers a rapid method for both quantitative determination of urinary phenols and preparation of phenols for chromatography.

## Method

Urine samples are prepared in triplicate. Two aliquots serve for quantitative measurement of phenols and one for chromatographic identification.

Ten milliliters of urine is saturated with 3 gm. of NaCl and ad-

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justed to *p*H 8.0 with 2% NaOH. Using an electric stirrer, this mixture and 25 ml. of anhydrous ether are mixed in a 200-ml. centrifuge jar for 10 min. The sample is then centrifuged at 2000 rpm for 5 min. The ether layer is pipetted off into a separatory funnel. This extraction is repeated three times, but the mixing period is reduced to 5 min. for the second and third extractions. The combined ether extracts are washed three times with 5 ml. of distilled $H_2O$, and this $H_2O$ wash is added to the residual urine from the *p*H 8 fraction.

At this point the ether extract of the third aliquot is set aside and allowed to evaporate to dryness. The other two aliquots are treated separately in the following manner.

The washed ether extract is emptied into a 250-ml. round-bottom flask which contains 10 ml. of distilled $H_2O$. Using a flash evaporator the ether phase is evaporated, leaving the 10 ml. of $H_2O$. To measure the amount of phenols extracted, 1 ml. of the aqueous phase is placed in a 25-ml. graduated cylinder and diluted with $H_2O$ to 10 ml. One milliliter of Folin-Ciocalteau reagent and 2 ml. of 20% $Na_2CO_3$ are added and the cylinders placed in boiling $H_2O$ for 1 min. The cylinders are allowed to cool, and then $H_2O$ is added to 25 ml. The blue color that develops is read on a photoelectric colorimeter with a red filter (wavelength range of 640–700 m$\mu$) and compared to that produced by a phenol standard (10 mg./100 ml.). This fraction represents the free phenols, ether extractable at *p*H 8 (*p*H 8 fraction).

The residual urine combined with the water washings of the previous ether phases are then brought to *p*H 1 with 1N $H_2SO_4$ and immediately extracted in the same way. The color is developed and read with *p*-hydroxy phenylacetic acid (10 mg./100 ml.) as standard. This fraction consists of the aromatic acid phenols (*p*H 1 fraction).

The residual urine at *p*H 1 is then refluxed with an equal volume of 1N $H_2SO_4$ for 1 hr. It is then re-extracted at *p*H 1 or less. Color is developed and read as for the *p*H 1 fraction. This fraction contains the conjugated phenols (hydrolyzed fraction). Our fractions *p*H 1 and hydrolyzed, together, are similar to Volterra's fraction 2, aromatic hydroxy acids.

## Results

### Recovery of Phenols by the Described Method

Phenol (Baker's reagent, crystalline) freshly opened, and *p*-hydroxy phenylacetic acid (Nutritional Biochemicals) were added to urine in individual concentrations of 2.5 and 5.0 mg./100 ml. Recov-

**Table 1.** Recovery of Added Phenol and *p*-Hydroxy Phenyl Acetic Acid

| *pH 8* | *pH 1* |
|---|---|
| 2.5 mg./100 ml. of each | |
| 96.5% | 81.5% |
| 106.8 | 81.5 |
| 120.6 | 81.5 |
| 106.8 | 78.9 |
| 107.2 | 86.6 |
| 5 mg./100 ml. of each | |
| 105.5% | 80.9% |
| 96.3 | 96.8 |
| 107.4 | 103.1 |
| 90.7 | 96.8 |
| 96.3 | 84.1 |

eries of these added substances from five samples of the same urine were determined, using the ether extraction method described. At *p*H 8, 103% of added phenol is extracted and at *p*H 1, 87% (Table 1).

When phenols are added separately, it is found that extraction of free phenol is equally effective at *p*H 8 and *p*H 1, while *p*-hydroxy phenylacetic and pyruvic acids are not extractable at *p*H 8 but are maximally extractable at *p*H 1. Therefore, specificity is obtained by extracting free phenols at *p*H 8 followed by acid phenols at *p*H 1.

### Variations Between Duplicate Determinations of Urines

Ten duplicate determinations were analyzed to establish error of the method. These are summarized in Table 2. Most agree within 10%.

**Table 2.** Duplicate Determinations (mg./ml.)

| *pH 8* | | *pH 1* | | *Hydrolyzed* | | *Residual* | |
|---|---|---|---|---|---|---|---|
| 0.014 | 0.017 | 0.187 | 0.153 | 0.367 | 0.429 | 0.086 | 0.089 |
| 0.009 | 0.009 | 0.048 | 0.050 | 0.046 | 0.045 | 0.037 | 0.037 |
| 0.013 | 0.013 | 0.149 | 0.157 | 0.209 | 0.223 | 0.084 | 0.081 |
| 0.011 | 0.013 | 0.096 | 0.094 | 0.363 | 0.333 | 0.128 | 0.126 |
| 0.010 | 0.010 | 0.376 | 0.333 | 0.358 | 0.223 | 0.162 | 0.145 |
| 0.018 | 0.018 | 0.029 | 0.027 | 0.041 | 0.040 | 0.071 | 0.076 |
| 0.011 | 0.012 | 0.058 | 0.055 | 0.082 | 0.082 | 0.065 | 0.061 |
| 0.008 | 0.007 | 0.048 | 0.046 | 0.087 | 0.073 | 0.060 | 0.057 |
| 0.005 | 0.010 | 0.338 | 0.312 | 0.151 | 0.165 | 0.070 | 0.066 |
| 0.007 | 0.006 | 0.045 | 0.047 | 0.112 | 0.101 | 0.143 | 0.107 |

Greater variations in the *p*H 8 fraction may be attributed to the very low concentration of phenols extractable at this *p*H. Differences greater than 15% are repeated as the extraction is considered inadequate.

### Comparison of Current Method with Steam Distillation Method of Volterra

Six urine specimens were analyzed for phenols by the steam distillation method (A) of Volterra's and by the method described in this report (B). The results are compared in Table 3. Volterra steam-distills urine that is slightly alkaline. This fraction he calls the "free volatile phenols." After acidification, the sample is redistilled and the quantity of phenols determined. This fraction represents the volatile conjugated phenols. The residue is then ether extracted, and these phenols Volterra calls the "aromatic hydroxy acids." Since this follows some hydrolysis, this fraction is similar to our *p*H 1 (aromatic acid phenols) and hydrolyzed fractions (conjugated phenols). While the three fractions obtained in each of the two methods cannot be compared directly, the total phenol extraction may be compared. In all instances, more phenols were extracted using the described method.

### Paper Chromatography of Phenolic Fractions

As described in the method section, the ether extracts from the third aliquot are used for chromatographic purposes. After the ether has evaporated, the residue is taken up in 95% ethanol and spotted on Whatman No. 1 paper. An ascending two-way system is used with isopropanol, 200 ml., ammonia, 10 ml., and water, 20 ml. as solvent I

**Table 3.** Comparison of Extraction Method (A) with Steam Distillation Method (B) (mg./ml.)

| *Urine specimen* | *pH 8** (*A*) | *I†* (*B*) | *pH 1** (*A*) | *Hyd** (*A*) | *II†* (*B*) | *Resid** (*A*) | *III†* (*B*) |
|---|---|---|---|---|---|---|---|
| 1 | 2.52 | 1.93 | 8.82 | 14.8 | 5.07 | 6.93 | 4.52 |
| 2 | 1.74 | 2.87 | 5.43 | 8.43 | 7.32 | 5.46 | 5.34 |
| 3 | 0.486 | 1.66 | 0.756 | 3.78 | 3.36 | 3.51 | 5.37 |
| 4 | 3.00 | 1.59 | 8.00 | 7.25 | 7.16 | 8.50 | 9.28 |
| 5 | 1.80 | 6.08 | 13.0 | 15.6 | 13.6 | | 6.48 |
| 6 | 2.99 | 2.47 | 75.9 | 11.0 | 33.6 | | 6.97 |

*Method A: *p*H 8: volatile free phenols; *p*H 1: free phenolic acids; Hyd: conjugated volatile phenols and phenolic acids; Resid: non-ether-soluble.

†Method B yields phenols as follows: Fraction I: volatile phenols and volatile conjugated phenols; Fraction II: phenolic acids and conjugated phenolic acids; Fraction III: non-extractable.

and benzene, 125 ml., acetic acid, 72 ml., and water, 3 ml. as solvent II (*5*).

Considerable variation was noted in the distinction and relative intensity of spots from the *p*H 1 fraction. The corresponding hydrolyzed fraction usually showed the same compounds but less intensively. No demonstrable phenolic compounds were found in the *p*H 8 chromatogram when diazotized sulfanilic acid and aroyl-glycine reagent were used as indicators.

Phenols excreted by children consisted chiefly of *p*-OH phenylacetic acid, *p*-OH phenyl lactic acid, m-OH benzoic acid, and an unidentified spot near *p*-OH phenylacetic acid which is yellow with sulfanilic acid.

Small amounts of *p*-OH phenylpyruvic, vanillic, and ferulic acids were also noted.

The *p*H 1 fraction of urines from the phenylketonuric acid contained mainly *p*- and *o*-hydroxyphenolic compounds. The quantitative variation recorded was not reflected in the apparent intensity of phenolic staining spots.

**Table 4.** PHENOL

| *Case No.* | *Diagnosis* | *Age* (yr.)* | *Wt.* (kg.)* | *Vol.* | *pH 8* | | |
|---|---|---|---|---|---|---|---|
| | | | | | *mg./day* | *mg./kg.* | *mg./creat.* |
| 1 | Retardation | ¾ | 8 | 250 | 4.6 | 0.6 | .13 |
| 2 | Bronchitis | 2 | 13 | 220 | 2.2 | 0.2 | .02 |
| 3 | Well | 3 | 16 | 500 | 3.5 | 0.2 | .01 |
| 4 | Well | 3 | 16 | 375 | 4.5 | 0.3 | .03 |
| 5 | Retardation | 3 | 13 | 470 | 4.2 | 0.3 | .05 |
| 6 | Well | 4 | 15 | 500 | 5.0 | 0.3 | .08 |
| 7 | Cardiac | 5 | 27 | 380 | 4.6 | 0.2 | .02 |
| 8 | Epilepsy | 7 | 23 | 180 | 3.1 | 0.1 | .01 |
| 9 | Bronchitis | 7 | 24 | 700 | 6.3 | 0.3 | .02 |
| 10 | Post polio | 7 | 23 | 375 | 5.2 | 0.2 | .02 |
| 11 | Scoliosis | 8 | 22 | 450 | 5.4 | 0.2 | .03 |
| 12 | Pneumonia | 8 | 20 | 575 | 5.2 | 0.3 | .02 |
| 13 | Neuritis | 9 | 30 | 500 | 5.0 | 0.2 | .01 |
| 14 | Cerebral palsy | 9 | 23 | 660 | 16.5 | 0.7 | .08 |
| 15 | Calcinosis | 9 | 26 | 600 | 6.6 | 0.2 | .02 |
| 16 | Well | 10 | 35 | 500 | 5.5 | 0.2 | .01 |
| 17 | Post polio | 10 | 41 | 1,500 | 10.5 | 0.3 | .02 |
| 18 | Neuritis | 13 | 36 | 470 | 6.6 | 0.2 | .01 |
| 19 | Well | 14 | 46 | 1,300 | 7.8 | 0.2 | .01 |
| 20 | Scoliosis | 14 | 52 | 500 | 4.1 | 0.1 | .01 |
| | MEAN | | | | 5.8 | 0.26 | 0.03 |
| | ± S.D. | | | | ± 3.1 | ± 0.14 | ± 0.03 |

*Adjusted to nearest Kg and year.

### Phenol Excretion in Children (Healthy and Ill)

Twenty-four-hour urines were collected under toluene from 20 children (9 months to 14 years in age). Four were well and the other 16 were hospitalized for varying causes. They received no unusual diets or drugs. Data from all are similar and are therefore combined.

Phenolic acids extracted at *p*H 1 varied from 5.8 in the 9-month infant to 70.5 mg. per day in a 10 year old (Table 4). The mean daily excretion was 29.5 ± 14.5 mg. per day in the children studied. When expressed on a weight basis, urinary phenolic acids averaged 1.3 ± 0.5 mg./kg. per day, of 0.12 ± 0.06 mg. per milligram of creatinine.

The free phenols (*p*H 8) were excreted in small quantities (Table 4). They averaged 5.8 ± 3.0 mg./24 hr., or 0.26 ± 0.14 mg./kg. per day, or 0.03 ± 0.03 mg. per milligram creatinine.

Conjugated phenols measured after hydrolysis averaged 48.5 ± 31.0 mg. per day, 1.9 ± 0.8 mg./kg. per day, or 0.18 ± 0.09 mg. per milligram of creatinine.

EXCRETION OF CHILDREN

| *pH 1* | | | *Hydrolyzed* | | | *Total* | | *Residual* |
|---|---|---|---|---|---|---|---|---|
| *mg./day* | *mg./kg.* | *mg./creat.* | *mg./day* | *mg./kg.* | *mg./creat.* | *mg./day* | *mg./kg.* | *mg./day* |
| 7.4 | 1.0 | .21 | 10.4 | 1.3 | .30 | 22.4 | 2.9 | 18.8 |
| 22.7 | 1.8 | .24 | 39.6 | 3.2 | .42 | 64.5 | 5.2 | 18.7 |
| 23.5 | 1.5 | .10 | 40.0 | 2.5 | .17 | 67.0 | 4.2 | 29.0 |
| 21.4 | 1.4 | .15 | 30.8 | 1.9 | .22 | 56.6 | 3.6 | 23.6 |
| 23.5 | 1.8 | .25 | 21.2 | 1.7 | .23 | 48.9 | 3.8 | 22.5 |
| 8.5 | 0.6 | .14 | 14.0 | 1.0 | .23 | 27.5 | 1.9 | 15.5 |
| 23.9 | 0.9 | .12 | 31.5 | 1.2 | .16 | 60.0 | 2.2 | 24.7 |
| 17.1 | 0.8 | .05 | 26.6 | 1.2 | .07 | 46.8 | 2.0 | 15.8 |
| 32.9 | 1.4 | .09 | 48.3 | 2.0 | .13 | 87.5 | 3.7 | 37.8 |
| 36.0 | 1.6 | .13 | 64.5 | 2.8 | .23 | 105.8 | 4.7 | 26.2 |
| 30.2 | 1.4 | .20 | 45.0 | 2.1 | .29 | 80.6 | 3.6 | 27.4 |
| 43.7 | 2.2 | .15 | 47.2 | 2.4 | .15 | 96.0 | 4.8 | 68.4 |
| 22.5 | 0.8 | .05 | 34.5 | 1.2 | .07 | 62.0 | 2.1 | 42.5 |
| 27.1 | 1.2 | .12 | 50.2 | 2.1 | .21 | 93.7 | 4.0 | 37.0 |
| 47.6 | 1.9 | .13 | 62.4 | 2.4 | .24 | 116.6 | 4.5 | 45.8 |
| 22.6 | 0.6 | 0.5 | 94.2 | 2.7 | .27 | 122.3 | 3.5 | 33.1 |
| 70.5 | 1.7 | .11 | 145.5 | 3.6 | .36 | 226.5 | 5.6 | 93.0 |
| 45.6 | 1.3 | .08 | 55.5 | 1.5 | .15 | 107.6 | 3.0 | 47.0 |
| 16.9 | 0.4 | .03 | 29.9 | 0.7 | .07 | 54.6 | 1.2 | 35.1 |
| 47.8 | 0.9 | .07 | 79.0 | 1.5 | .15 | 130.1 | 2.5 | 40.8 |
| 29.6 | 1.3 | .12 | 48.5 | 1.9 | .18 | 83.8 | 3.4 | 35.1 |
| ± 14.5 | ± 0.5 | ± .06 | ± 31.0 | ± 0.8 | ± 0.09 | ± 45.0 | ± 1.2 | ± 18.3 |

**Table 5.** PHENOL EXCRETION IN PHENYLKETONURIA

| *12-hr. period* | *Phenylalanine intake mg./kg./day* | *mg./kg./12 hr.* | | | |
|---|---|---|---|---|---|
| | | *pH 8* | *pH 1* | *Hyd* | *Res* |
| 1. A.M. | 19 | 0.27 | 1.2 | 1.6 | 1.3 |
| 2. P.M. | 20 | 0.07 | 0.4 | 0.4 | 0.6 |
| 3. A.M. | 20 | 0.20 | 1.3 | 1.0 | 1.3 |
| 4. A.M. | 26 | 0.14 | 1.7 | 1.5 | 1.5 |
| 5. P.M. | 28 | 0.04 | 0.3 | 0.3 | 0.4 |
| 6. A.M. | 28 | 0.20 | 2.0 | 1.7 | 1.3 |
| | | *mg./kg. per day* | | | |
| Period 2 + 3 | 20 | 0.27 | 1.7 | 1.4 | 1.9 |
| Period 5 + 6 | 28 | 0.24 | 2.3 | 2.0 | 1.7 |

Total phenols excreted were thus 83.8 ± 45.0 mg. per day or 3.4 ± 1.2 mg./kg. per day.

Residual Folin-Ciocalteau reacting material which was not extracted and is presumably tyrosine-like material averaged 35.0 ± 18.3 mg. per day.

### Phenol Excretion in Phenylketonuria

A 2-year-old male phenylketonuric weighing 12.7 kg., who had been maintained on a limited phenylalanine intake since 2 months of age, was hospitalized. He was fed a measured diet containing increasing amounts of phenylalanine (240–360 mg. per day). Consecutive 12-hr. urines were collected after 48 hr. of each diet. No phenylalanine was fed during the night collection period. Phenolic fractions for several of these 12-hr. urines are recorded in Table 5. For purposes of comparison, 24-hr. excretions were calculated by combining data from two consecutive 12-hr. collections. The *p*H 1 fraction, which contains the free phenolic acids, shows a response to the increased phenylalanine intake. The daytime collection appears to be more sensitive to this metabolic manipulation than the total 24-hr. intake. At 360 mg. phenylalanine intake (28.4 mg./kg. body weight) the *p*H 1 fraction significantly exceeds normal excretion values.

## Discussion

A method for the determination of urinary phenols, using ether extraction, is described. Recovery of added phenol and *p*-hydroxy phenylacetic acid is at least 79%; *p*-hydroxy phenylpyruvic acid, however,

is very slightly soluble in ether. Duplicate determinations agree within 10%. Simultaneous fractions for chromatography are made available.

Total phenolic excretion in the 20 children studied was 3.4 ± 1.2 mg./kg. per day. Using steam distillation, Volterra detected 2 mg./kg. per day of phenols in adults. Presumably the steam distillation method does not extract the phenols as thoroughly as ether.

Rogers *et al.* (*3*) used the method of Schacter, which is closest to that described here. Urine is hydrolyzed first, followed by continuous ether extraction. Fractions are then separated with $NaHCO_3$ and NaOH. Color is developed with Millon's reagent. If one assumes their patients weighed 70 kg., their excretion rates would be: aromatic hydroxy acids 2 mg./kg., volatile phenols, 3 mg./kg., and ether-insoluble residue, 1.5 mg./kg. per day. Their method does not distinguish between conjugated and nonconjugated phenols.

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# Relationship of Serum and Urinary Proteins in Neoplastic States: A Preliminary Survey

*A. Nettleship, James A. Strother, and H. Siemsen Smith*

**Twenty-four-hour urine specimens from patients with and without malignancies were concentrated by dialysis. The electrophoretic mobility of the urine protein components was compared with that of the respective serum proteins. Control values were similar to those established in the literature. Urinary protein concentrations found in malignant states were higher. Generally, there is a positive correlation between serum concentration of globulins and urinary excretion. It is not possible to prove in either controls or in patients bearing malignancies that the amount of urinary protein excreted depends upon molecular size.**

THE FINDING of high serum globulin values in patients with various malignancies (*1*) caused us to consider the possibility of finding these proteins in the urine of such patients. The normal kidney is an excellent conserver of plasma proteins. Although the urines of these patients were negative for protein by ordinary laboratory tests, we sought to extend our methods and search more thoroughly in their urine for proteins. It is ordinarily stated that pathologic proteinuria from diseased kidneys allows selective plasma protein escape according to molecular weight (*2, 3*). The usual assumption is that the urine simply reflects the albumin/globulin ratio of the plasma (*2*), in spite of studies which point to macroglobulinemia, including the M-protein of multiple myeloma, as being important in producing proteinuria not related to the ratio of albumin globulin molecular weights (*4, 5*).

We were also encouraged, by the findings of Hammack (*6*) and others (*4, 5*), to see if this reflected itself in a urinary component in the urine of some of our patients with malignancies. If their values were

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shown to be significant, even though the amounts of proteins might be small, this would offer another method of characterizing neoplastic growth.

## Method

Following a preliminary survey on first morning specimens obtained from normal patients, 24-hr. urine samples were collected in 4-L. jugs containing 5 ml. toluene as preservative. As soon as the specimen reached the laboratory and the volume was measured, it was refrigerated at 4°. A 400-ml. aliquot of the centrifuged specimen was concentrated by dialysis through a cellulose membrane with an average pore diameter of 24 Å, against 25% polyvinyl pyrrolidone at 4°. Dialysis was continued at 4° until the specimen was reduced to approximately 2 ml. and the exact volume measured to the nearest tenth of a milliliter. This procedure required 24–48 hr. Total proteins were determined on the concentrates by the Biuret method (*7*). The 24-hr. total protein excretions were calculated for each patient. The electrophoretic mobility of the protein components within the urine was determined using a Durrum-type cell (Spinco) (*8*). Depending on the total protein concentration, 10–30 $\mu$l. of the concentrates were applied to paper strips using veronal buffer, pH 8.6, ionic strength 0.075, and a current of 2.5 ma. was applied for 16 hr. The strips were heated, then stained with bromophenol blue and scanned with a densitometer (Spinco Analytrol). The concentrations of each component were calculated from the area under the electrophoretogram tracing, using the total protein value as assayed by the chemical procedure.

In order to establish the chemical nature of the nonmigrating fraction, the following color reactions were performed upon this area on the filter paper and cellulose-nitrate strips: bromphenol blue, amidoschwarz, ponceau S, nigrosin, xanthoprotein, and ninhydrin.

## Results and Discussion

The initial preliminary survey was made on 6 healthy individuals, and a 24-hr. excretion volume was estimated to be 1250 ml. Under these circumstances, average 24-hr. protein excretion was found to be 31 mg. The subsequent study was undertaken on 10 chronically ill patients in which actual measurement of the 24-hr. specimens was made. These controls showed an average 24-hr. total protein excretion of 68.3 mg. In addition to the preliminary survey and study of

**Table 1.** URINARY PROTEIN LEVELS IN NONMALIGNANCY

| | | Urine | | | | | | | | |
|---|---|---|---|---|---|---|---|---|---|---|
| *Patient* | *Serum T. P. (gm./100 ml.)* | *mg./24 hr.* | *24-hr. vol. (ml.)* | *Alb. (mg.)* | *$\alpha_1$ (mg.)* | *$\alpha_2$ (mg.)* | *$\beta$ (mg.)* | *$\gamma$ (mg.)* | *Nonmig. prot. (mg.)* | *Clinical diagnosis* |
| J.D. | 7.4 | 49.5 | 1850 | 3.3 | 3.3 | 9.9 | 9.9 | 6.6 | 16.5 | Remove callus; pneumonitis, rt. |
| R.W. | 6.6 | 44.7 | 2280 | 0.0 | 0.0 | 5.6 | 11.2 | 14.0 | 14.0 | Fracture—residual |
| G.G. | 6.4 | 52.0 | 2420 | 6.5 | 9.8 | 9.8 | 9.8 | 8.1 | 8.1 | Cervical spine arthritis |
| G.R. | 7.4 | 98.3 | 1260 | 40.7 | 8.0 | 9.0 | 12.9 | 11.8 | 15.9 | Chr. cholecystitis and cholelithiasis |
| H.L. | 6.9 | 110.9 | 1420 | 15.9 | 10.1 | 11.0 | 26.4 | 21.1 | 26.4 | Lumbar disc syndrome |
| C.R. | 6.3 | 60.0 | 2000 | 12.0 | 3.0 | 9.0 | 9.0 | 12.0 | 15.0 | Rheumatoid arthritis |
| D.S. | 6.6 | 54.9 | 1900 | 3.2 | 6.5 | 9.7 | 9.7 | 6.5 | 19.3 | Arteriosclerotic heart disease |
| E.T. | 6.3 | 47.8 | 1540 | 17.9 | 6.0 | 10.0 | 8.0 | 4.0 | 2.0 | Old gunshot wound |
| F.C. | 6.6 | 120.1 | 2390 | 16.0 | 16.0 | 16.0 | 32.1 | 16.0 | 24.0 | Inguinal hernia |
| A.R. | 7.6 | 44.9 | 1330 | 8.4 | 3.0 | 7.8 | 7.8 | 8.4 | 9.5 | Peptic ulcer |
| mg./avg. 24 hr. | | 68.3 | | 12.4 | 6.8 | 9.8 | 13.7 | 10.9 | 15.1 | |
| % avg. | | 100 | | 18.0 | 9.9 | 14.2 | 20.0 | 15.9 | 22.0 | |
| A/G ratio | | | | 18% | | | 82% = | $\frac{18}{82}$ = | 0.22 | |
| Extremes | | 44.7— | | 0— | 0— | 5.6— | 7.8— | 4.0— | 2.0— | |
| Mg./24 hr. | | 120.1 | | 40.7 | 16.0 | 16.0 | 26.4 | 21.1 | 26.4 | |

Bence-Jones protein determinations on urine specimens were negative.

10 control patients, 9 patients with malignancies were studied. The results of these surveys are given in Tables 1–4 and in Fig. 1.

The values for normal urinary protein excretion as reported in the literature vary widely. Recent values obtained using electrophoresis technic (*9*) are 0.03–1.0 mg./kg. body weight per day, which, assuming a body weight of 70 kg. and 1250 ml. of 24-hr. urine excretion, would give 2.1–70 mg./24 hr. In the studies of normal and nonmalignant cases, these figures were duplicated. On the other hand, the urine of patients having a malignant disease show, by these criteria, true proteinemia. The protein fractions largely responsible for this are the

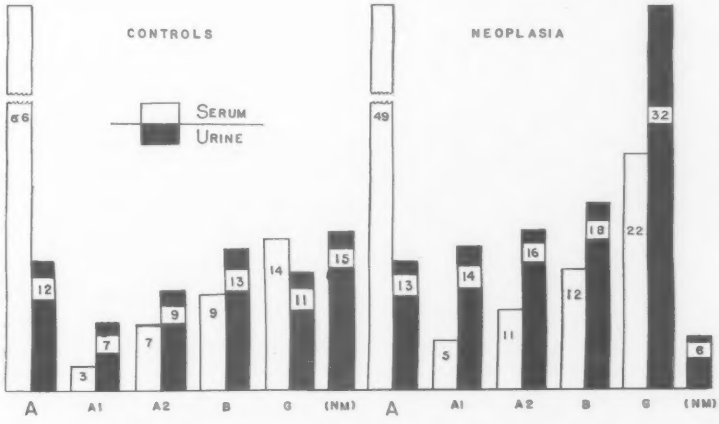


**Fig. 1.** Serum versus urine proteins. Values are in percentages.

globulins, and this correlates well with the increase in the plasma globulins found in malignancy. However, the quantity excreted via the urine is not directly proportional to the serum globulin increase. One of the most striking phenomena connected with this study was the finding of nonmigrating proteins in both series. These proteins may be of such a sensitive nature that in spite of our best efforts and mildest laboratory conditions, they became denatured. This group is electrophoretically neutral and remains at the site of application. This finding also suggests that they may be cryoglobulins, perhaps in the mucoprotein class. Further studies in this area with six different tests for proteins, as listed under *Method*, showed that they were all positive for proteins. Thus, the nonmigrating fractions, as shown by

**Table 2.** URINARY PROTEIN LEVELS IN MALIGNANCY

| | | Urine | | | | | | | | |
|---|---|---|---|---|---|---|---|---|---|---|
| *Patient* | *Serum T. P. (gm./100 ml.)* | *mg./24 hr.* | *24-hr. vol. (ml.)* | *Alb. (mg.)* | *$\alpha_1$ (mg.)* | *$\alpha_2$ (mg.)* | *$\beta$ (mg.)* | *$\gamma$ (mg.)* | *Nonmig. prot. (mg.)* | *Clinical diagnosis* |
| J.B. | 6.1 | 145.9 | 400 | 3.5 | 14.3 | 35.6 | 39.1 | 21.9 | 32.1 | Adenocare. colon |
| F.S. | 6.3 | 81.6 | 296 | 6.2 | 11.7 | 18.4 | 12.9 | 24.7 | 7.5 | Care. stomach |
| D.J. | 5.9 | 286.7 | 380 | 2.2 | 31.0 | 76.8 | 79.1 | 95.5 | 2.3 | Care. colon |
| E.K. | 5.7 | 107.3 | 980 | 66.1 | 4.3 | 5.9 | 13.2 | 16.2 | 1.4 | Bronchogenic care. |
| J.T. | 6.7 | 792.0 | 400 | 39.6 | 158.4 | 79.2 | 158.4 | 297.0 | 59.4 | Bronchogenic care. |
| R.H. | 4.5 | 82.4 | 900 | 12.4 | 7.7 | 14.0 | 14.0 | 16.1 | 18.0 | Lymphosarcoma |
| W.L. | 6.1 | 674.0 | 722 | 64.7 | 103.0 | 114.6 | 95.0 | 296.6 | 0.0 | Lymphosarcoma |
| H.H. | 7.8 | 302.0 | 1325 | 119.6 | 34.1 | 51.3 | 45.3 | 45.3 | 6.0 | Lymphocytic leuk. |
| L.V. | 6.4 | 67.2 | 255 | 14.7 | 4.0 | 8.1 | 12.1 | 12.1 | 16.1 | Care. stomach |
| mg./24 hr. avg. | | 282.0 | | 36.6 | 40.9 | 44.8 | 52.1 | 91.7 | 15.9 | |
| % avg. | | 100 | | 13 | 14.5 | 15.9 | 18.5 | 32.5 | 5.6 | |
| A/G ratio | | | | 13% | | 87% = | $\frac{13}{87}$ | = 0.15 | | |
| Extremes Mg./24 hr. | | 67.2— 792.0 | | 2.2— 119.6 | 4.0— 158.4 | 5.9— 114.6 | 12.1— 158.4 | 12.1— 297.0 | 0— 59.4 | |

Bence-Jones protein determinations were made on urine specimens, except for R.H.; all were negative except for W. L, with faintly positive result.

**Table 3.** Blood Serum Proteins in Nonmalignancy

| *Patient* | *Alb. (gm.)* | *$\alpha_1$ (gm.)* | *$\alpha_2$ (gm.)* | *$\beta$ (gm.)* | *$\gamma$ (gm.)* | *Clinical diagnosis* |
|---|---|---|---|---|---|---|
| J.D. | 4.3 | 0.2 | 0.4 | 0.6 | 1.1 | Remove callus; pneumonitis, rt. |
| R.W. | 5.0 | 0.2 | 0.3 | 0.6 | 0.5 | Fracture—residual |
| G.G. | 4.8 | 0.2 | 0.3 | 0.6 | 0.5 | Cervical spine arthritis |
| G.R. | 4.7 | 0.1 | 0.5 | 0.8 | 1.3 | Chr. cholecystitis; cholelithiasis |
| H.L. | 4.1 | 0.2 | 0.7 | 0.9 | 1.0 | Lumbar disc syndrome |
| C.R. | 4.2 | 0.2 | 0.6 | 0.5 | 0.8 | Rheumatoid arthritis |
| D.S. | 4.7 | 0.2 | 0.3 | 0.5 | 0.9 | Arteriosclerotic heart disease |
| E.T. | 4.2 | 0.2 | 0.6 | 0.5 | 0.8 | Old gunshot wound |
| F.C. | 4.8 | 0.2 | 0.3 | 0.6 | 0.7 | Inguinal hernia |
| A.R. | 3.9 | 0.2 | 0.4 | 0.7 | 2.4 | Peptic ulcer |
| Average (%) | 66.8 | 2.6 | 6.6 | 9.6 | 14.4 | |

these technics, have a protein component which has an affinity for the dye and which leaves a dark band at the application point. This artifact must then be mildly denatured protein, an idea which is confirmed by the finding that the amounts of nonmigrating fraction are increased with the length of time the protein is held at 4°.

Bence-Jones protein determinations were performed on these urine specimens with negative results in all except one case, patient W.E.L. His urinary protein was extremely high—674 mg./24 hr., and gave a faintly positive test. This finding correlates with what had previously been shown for a similar case of the lymphoma group (*10, 11*).

**Table 4.** Blood Serum Proteins in Malignancy

| *Patient* | *Alb. (gm.)* | *$\alpha_1$ (gm.)* | *$\alpha_2$ (gm.)* | *$\beta$ (gm.)* | *$\gamma$ (gm.)* | *Clinical diagnosis* |
|---|---|---|---|---|---|---|
| J.B. | 3.0 | 0.3 | 0.7 | 0.8 | 1.3 | Adenocarc. colon |
| F.S. | 3.3 | 0.3 | 0.8 | 1.0 | 0.9 | Carc. stomach |
| D.J. | 3.0 | 0.4 | 0.9 | 0.7 | 0.8 | Carc. colon |
| E.K. | 3.5 | 0.3 | 0.6 | 0.5 | 0.8 | Bronchogenic carc. |
| J.T. | 3.0 | 0.3 | 0.5 | 0.8 | 2.1 | Bronchogenic carc. |
| R.H. | 2.3 | 0.3 | 0.5 | 0.5 | 0.9 | Lymphosarc. |
| W.L. | 3.0 | 0.4 | 0.9 | 0.8 | 1.0 | Lymphosarc. |
| H.H. | 4.4 | 0.2 | 0.5 | 0.8 | 1.9 | Lymphocytic leuk. |
| L.V. | 1.9 | 0.2 | 0.5 | 0.7 | 3.1 | Carc. stomach. |
| Average (%) | 49.9 | 5.2 | 10.7 | 11.7 | 22.8 | |

From the statistical data contained in this survey, it is obvious that there is a difference between normal kidney excretion of abnormal plasma proteins and diseased kidney excretion of plasma proteins. Since the globulins have the greater molecular weight, they should be excreted in lesser amounts than the albumin (*6*), yet under the circumstances of the experiment above, the opposite was proved to be true. Not only did the globulins appear in larger amounts (Fig. 1), although present in lesser concentrations in the blood plasma, but, also, in every fraction with the exception of gamma globulin in the control series, individual globulins were present in the urine in greater amounts than in the blood plasma. If further work substantiates these findings, a new theory will have to be formulated to cover the facts. As of now, when trace amounts are considered, the normal kidney in both control and malignant states releases larger amounts of globulin than albumin, and the individual globulins are not excreted according to their plasma protein concentrations.

It may be noted that our data also consider the nonmigrating protein (Fig. 1). In the method used, every care was taken to handle the urinary protein under the mildest conditions in order to minimize denaturation. In spite of this, the presence of the nonmigrating fraction suggests that there may be some slight denaturation. However, even if this protein is placed in any one globulin category, it still will not change their fundamental relations nor the conclusions.

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# A Simple Low-Voltage Paper Electrophoretic Method for the Determination of Urinary Vanillylmandelic Acid (VMA)

*F. Eichhorn and A. Rutenberg*

**A simple and accurate method for the determination of urinary 3-methoxy-4-hydroxy-mandelic acid, vanillyl-mandelic acid (VMA), is described. The separation of VMA from all other phenolic acids in approximately 0.05 ml. untreated urine is achieved by using low-voltage paper electrophoresis with different buffer concentrations for the cathode and the anode. The concentration of the electrophoretically separated VMA is determined colorimetrically in the eluate. Values of excreted VMA in 24-hr. normal urine and those from subjects with pheochromocytoma differ considerably. We are now applying this new principle of "two solutions paper electrophoresis" for better separation of amino acids, proteins, and other biologic mixtures.**

Recent publications emphasize the importance of urinary 3-methoxy-4-hydroxy mandelic acid (vanillylmandelic acid) in the diagnosis of phaeochromocytoma (*1–5*). Many methods have been suggested for its detection, but none lends itself to rapid routine investigations with a large number of urine samples.

Looking for a reliable and quick procedure, we applied a new method of "two-solution paper electrophoresis" using different concentrations of buffer for each pair of the electrode compartments. Using low-voltage electrophoresis, we were able to determine VMA quantitatively with about 0.05 ml. of urine. We found concentrations consistent with the results of Klein and Chernaik (*6*), who used urine extracts in low-voltage "one-solution electrophoresis."

## Method

A drop of the untreated urine is applied to a filter paper strip and placed in an electrophoretic apparatus which contains two solutions of

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different concentrations in each pair of the electrode compartments. The electrophoresis is run overnight. It results in separation of VMA from all other phenolic acids, VMA appearing as a purple band after staining of the strip with a diazo mixture. The band is eluted and read in a colorimeter.

### Apparatus

1. Paper electrophoresis apparatus
2. Filter paper strips (Schleicher and Schüll 2043 b mgl.)
3. Rectifier
4. Klett-Summerson colorimeter coupled to a Kipp galvanometer (*7*)
5. Electric hair dryer

### Reagents

1. VMA standard: 12.5 mg./100 ml. in distilled water slightly acidified with HCl (2.5 $\mu$g. in 0.02 ml.) (This must be kept refrigerated in a dark bottle.)
2. Solutions for electrophoresis: (a) 2.5% acetic acid for the cathode compartments; (b) 0.75% acetic acid for the anode compartments
3. p-nitroaniline solution: 0.1 gm. dissolved in 2 ml. conc. HCl and made up to 100 ml. with water (Kept in refrigerator.)
4. Sodium nitrite: 0.2% w/v, prepared fresh once a week (Kept in refrigerator.)
5. Potassium carbonate: 10% w/v in water (Kept in refrigerator.)
6. Diazo reagent: 7.5 ml. of Reagent 3 mixed with 10 ml. of Reagent 4 and, after 1 min., with 20 ml. of Reagent 5 (Make fresh just before use and keep refrigerated during dyeing of the strips.)
7. Methanol, anhydrous
8. Sodium carbonate: 2% w/v in water
9. Alkaline methanol solution: 2 parts of Reagent 7 added slowly to one part of Reagent 8 before use

### Procedure

Spot 1/20,000 part of a 24-hr. urine sample* to the middle of an electrophoretic paper strip as a streak of 2 cm. across the width of the

*In order to obtain comparable values of VMA in 24-hr. urines, only 1/20,000 part of the 24-hr. urine is spotted on the filter paper. (For example, when the quantity of 24-hr. urine is 2000 ml., 0.1 ml. is applied; when it is 500 ml., only 0.025 ml. urine is spotted.) The quantity (in milligrams) of VMA excreted in 24 hr. is obtained by multiplying the concentration of the eluted VMA band by 20. When only a small aliquot of urine is spotted, the phenolic acids move freely and separate well; no interference is caused by concentrated urine.

strip. Apply 0.02 ml. standard (Reagent 1) on a separate strip. The application is done with a current of hot air blowing from an electric hair dryer. Fill the cathode compartments of the electrophoretic apparatus with 2.5% acetic acid (Reagent 2a) and the anode compartments with 0.75% acetic acid (Reagent 2b). Moisten the strips at both ends with the corresponding solutions and place in the apparatus. When the paper strips are completely wet, apply a constant current of 1 ma for each strip overnight (about 17 hr.). At the end of this time, air-dry the strips and then heat for 30 min. at 80°. Prepare cooled diazo reagent (Reagent 6). Within 30 sec. dip the strips in the reagent. VMA appears as a clear purple band about 4 cm. from the application line towards the anode (Fig. 1). Cut out the VMA bands, which are

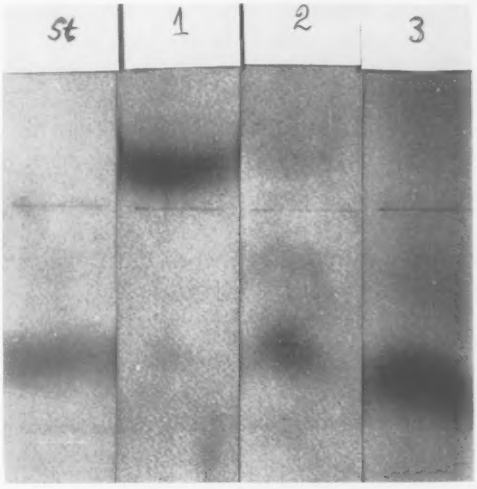


**Fig. 1.** *St*, standard 2.5 μg. VMA applied on paper strip (corresponds to 50 mg. in 24 hr.); *1*, normal urine—1.3 mg. VMA in 24 hr. (The strong violet band toward the cathode represents vanillic acid.); *2*, excretion of 30 mg. VMA in 24 hr.; *3*, excretion of 60 mg. VMA in 24 hr. Strips are oriented with cathode end at top, anode end at bottom.

identified by the position of the standard, while the paper strips are still moist, and place each in one test tube. Cut out a band of the same size from the dyed paper and place it in a separate test tube; this serves as zero blank. Add 4 ml. of alkaline methanol (Reagent 9) to each tube, keep in a dark place, shake occasionally during 30 min., and then read at 520 mμ in a photocolorimeter.

Calculation is as follows.

$$\frac{\text{Concentration of standard} \times \text{reading of test sample (unknown)}}{\text{Reading of standard}} \times 20 = \text{mg. VMA in 24-hr. urine sample}$$

When the urine subjected to electrophoresis produces a strong purple band at a position corresponding to that of the standard on the control strip, increased VMA is suspected and the urine should be run again overnight, once as before and once with an added standard (0.01 ml. = 1.25 VMA $\mu$g.). This will give the exact position of the VMA in this urine. After some experience the position of the purple band is clearly discerned in the pattern of all the phenolic acids appearing in urine, and can be detected easily.

We prefer a 24-hr. sample of urine in order to avoid an extra determination of creatinine.

No special precautions for collecting the urine are necessary. According to Studnitz (*8*) VMA is stable without acidification at room temperature for 8 days.

A very practical feature of this two-solution electrophoresis is that the same solutions may be used again for a few days, with a little acetic acid of the corresponding concentrations added to each compartment.

A similar principle of different solutions has been applied by Alexander Kolin (*9*) in Tiselius' moving boundary electrophoresis for proteins. In his system a *p*H gradient is used.

#### Preliminary Test (Spot Test)

When a large number of urines is to be examined, it is advisable to screen them in the following simple manner. Keep the patient on a diet 24 hr. before gathering the urine (no medication, no ingesting of fruit, coffee, tea, and vanilla). Place 1/50,000 part of a 24-hr. urine sample as a round spot on filter paper, dry in hot air, and dip through freshly prepared diazo mixture. Let dry in air and examine for color development after a few minutes. The resulting spots in normal urines after diet are light brown, with a faint purple tinge on the outer circle. These may be considered as negative and need not be examined by electrophoresis. If the spot develops a definite purple color, with a dominating intensity on the outer circle, the urines should be further examined by electrophoresis. The purple color may be due to VMA, vanillic acid, or o-hydroxy-phenyl acetic acid or other phenolic acids. Thus, when the patients are kept on the diet described above, the specificity of the purple tinge due to VMA is increased.

## Experience with New Method

This low-voltage electrophoretic method for VMA was used with 180 urine samples sent to our laboratory for estimation of catecholamines.

**Table 1.** PATHOLOGIC VMA LEVELS DETERMINED WITH LOW-VOLTAGE ELECTROPHORESIS METHOD

| *Condition* | *Remarks* | *VMA in 1 ml. urine (μg.)* | *VMA in 24-hr. urine (mg.)* | *Catechol-amines in 24-hr. urine (μg.)* | *VMA in 24-hr. urine after operation (mg.)* |
|---|---|---|---|---|---|
| Severe burn | 80% | 31 | | | |
| Suspected pheochromocytoma | Died after brain operation | 29 | 30 | 820 | |
| Shock | Received noradrenaline infusion | 165 | | | |
| Pheochromocytoma | Increased catecholamines found in our laboratory 3 yr. before. | 27 | 34 | 1450 | |
| Pheochromocytoma | VMA examined after 3-yr. storage in freezer. | 23 | 25 | 675 | |
| Pheochromocytoma (11 yr. old) | Urine supplied by another hospital | 39 | 35 | 3600 | Normal |
| Pheochromocytoma | | 30 | 35 | 880 | 2, 5 |
| Pheochromocytoma (pregnant) | Cesarean section performed in 8th month of pregnancy; tumor removed at same time (child and mother in good health). | 35 | 65 | 800 | 5 |

The following results for VMA excreted in 24 hr. were obtained: in a group of 110 patients, 0.8–4.9 mg.; in 37 patients, 5–7.9 mg.; 18 patients, 9–10 mg.; and in a group of 15 patients, 10–13 mg.

Table 1 gives data on pathologic results obtained.

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# Micromethod for Estimation of Serum Adenosine Deaminase

*Robert G. Martinek**

**A micromethod is described for assessing adenosine deaminase activity in serum. Its greater simplicity recommends it for routine use in the clinical chemistry laboratory. Comparison is made with a previous method described in this Journal. No clinical appraisal was made.**

A METHOD for the measurement of serum adenosine deaminase activity was recently published in this Journal (*1*). This method requires 2 ml. of serum, suffers from an unnecessarily large number of manipulations, and utilizes an unstable, relatively insensitive Nessler color reaction (*2*). Attempts to overcome these difficulties constitute the basis for this report.

A catalyzed indophenol reaction for the determination of ammonia, which is reportedly quite sensitive and produces a stable blue color, has been published by Chaney and Marbach (*3*). These authors have applied the reaction to the determination of urea after hydrolysis with urease, and reagents are available commercially. It is a modification of the Bertholet reaction (*4*). The following is a description of the application of this reaction to the determination of ammonia liberated by serum adenosine deaminase activity.

## Reagents

*Buffered substrate, pH 7.05* In a 100-ml. volumetric flask, dissolve 1.79 gm. of reagent-grade $Na_2HPO_4$ and 0.78 gm. of reagent-grade $KH_2PO_4$ in about 50 ml. of preboiled distilled water. Add 18 mg. of adenosine (free base), dilute to the 100-ml. mark with preboiled

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distilled water, and mix thoroughly until all solute is dissolved. This may be accelerated by heating the stoppered flask under hot tap water. Check the pH with a glass electrode, at 25°. It should be 7.05 ± 0.05; if not, remake the solution. Store in refrigerator, and prepare fresh every 3 months.

*Phenol color reagent** In a 500-ml. volumetric flask, place 5.4 ml. of liquefied phenol, U.S.P., "gilt label," and 25 mg. of reagent-grade sodium nitroferricyanide. Dilute to the 500-ml. mark with distilled water and mix thoroughly. Store in refrigerator. (This is stable at least 3 months.)

*Alkali-hypochlorite reagent** In a 500-ml. volumetric flask, place 62.5 ml. of 1 N sodium hydroxide and 4.2 ml. of 5% sodium hypochlorite solution (commercial bleach solution may be used). Dilute to the 500-ml. mark with distilled water and mix thoroughly. Store in refrigerator. (This is stable about 1 year.)

*Nitrogen standard* (1) Stock standard†—In a 250-ml. volumetric flask, place 0.3539 gm. of reagent-grade, anhydrous ammonium sulfate. Dilute to the 250-ml. mark with distilled water and mix thoroughly. One milliliter of this solution contains 0.3 mg. of nitrogen. (2) Working standard†—In a 100-ml. volumetric flask, pipet 20 ml. of the stock standard. Dilute to the 100-ml. mark with distilled water and mix thoroughly. One milliliter of this solution contains 0.06 mg. of nitrogen.

## Procedure

### Calibration Curve

In five 25-ml. volumetric flasks, place 5, 10, 15, 20, and 25 ml. of the working standard. Dilute to the 25-ml. mark, in each case, with distilled water. These solutions are equivalent to 12, 24, 36, 48 and 60 U. of adenosine deaminase activity, respectively. Use 0.05 ml. of each solution in place of serum in the routine assay that follows. Prepare a graph relating units of adenosine deaminase activity to absorbance. This should be checked at regular intervals. A typical calibration curve is shown in Fig. 1.

### Assay

Pipet 0.5 ml. of buffered substrate, pH 7.05, into each of 2 test tubes (15 × 125-mm.). Mark one tube "sample" and the other "blank."

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†Hartman-Leddon Co., Philadelphia, Pa.

Place both tubes in a 37° water bath for 2–3 min. Add to the sample tube 0.05 ml. of unhemolyzed serum or plasma. Stopper, mix gently, and replace in 37° bath. Exactly 60 min. after adding the serum, add 2.5 ml. of phenol color reagent to each tube. This stops the reaction.

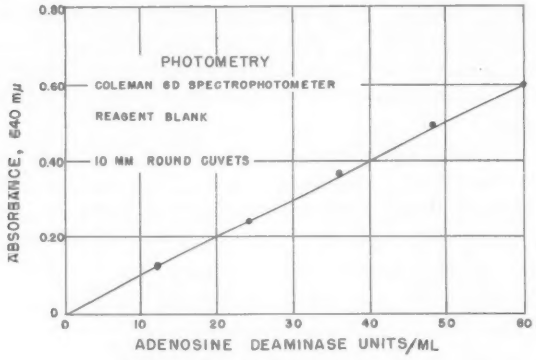


**Fig. 1.** Typical calibration curve.

To the blank tube, add 0.05 ml. of serum and to both tubes add 2.5 ml. of alkali-hypochlorite reagent. Stopper and promptly mix by inverting at least 3 times. Place both tubes in a heating block or water bath at 37° for 15 min. for color development. Readings are made in 10-mm. round cuvets in a spectrophotometer,* at 640 mμ, against the reagent blank adjusted to 100% transmittance. The color is stable for about 20 min. The units of adenosine deaminase activity are read from the calibration curve. One unit of adenosine deaminase activity is the amount that will liberate 1 μg. of ammonia nitrogen per milliliter of serum/plasma per hour at 37°. To convert these units to international units, multiply by 0.476 (*5*). For values over 70 U., the serum must be diluted with saline and the test repeated as reported by Koehler and Benz (*1*).

## Experimental

### Comparison of Methods

In order to validate this modification, the present method was compared with that of Koehler and Benz (*1*). The results are shown in Table 1.

*Coleman Junior, Model 6D, Coleman Instruments, Inc., Maywood, Ill.

**Table 1.** ASSAY RESULTS WITH TWO METHODS FOR SERUM ADENOSINE DEAMINASE

| *Test No.* | *Koehler-Benz method (1) (U./ml.)* | *Present method (U./ml.)* | *Difference* |
|---|---|---|---|
| 1 | 9 | 10 | 1 |
| 2 | 12 | 12 | 0 |
| 3 | 7 | 6 | 1 |
| 4 | 15 | 15 | 0 |
| 5 | 12 | 11 | 1 |
| 6 | 33 | 35 | 2 |
| MEAN | 15 | 15 | |

On a larger series, involving 20 serums analyzed in duplicate, the standard deviation, calculated by the formula (*6*):

$$\text{S.D.} = \sqrt{\frac{\Sigma\,(x_1 - x_2)^2}{N - 1}}$$

was 0.25 for the Koehler-Benz method and 0.20 for the present method.

### Stability of Adenosine Deaminase

Adenosine deaminase was found stable in serum at room temperature (25°–28°) for at least 3 days, but not 4 days, and in the refrigerator (approx. 4°) for at least 2 weeks. The enzyme has been reported stable in the freezer for over 6 months (*7*). Heparinized plasma may be used in place of serum. There appears to be some inhibition of adenosine deaminase activity in oxalated plasma.

### Spectral Characteristics of Reaction Mixture

Absorption spectra were determined on pooled serum and on a 58-U. standard (ammonium sulfate). Figure 2 is a graph of the results. Maximum absorption occurred at 640 m$\mu$.

### Chloroform as Preservative for Buffered Substrate

The effect of chloroform as a possible preservative for the buffered substrate, pH 7.05, was studied. One milliliter of chloroform was added to 100 ml. of buffered substrate and the mixture shaken. Analysis of pooled serum resulted in 9 U. of adenosine deaminase activity when no chloroform was used as a preservative in the buffered substrate and 10 U. of activity with chloroform as a preservative. This requires further study, and no recommendation for the use of chloroform is being made at this time.

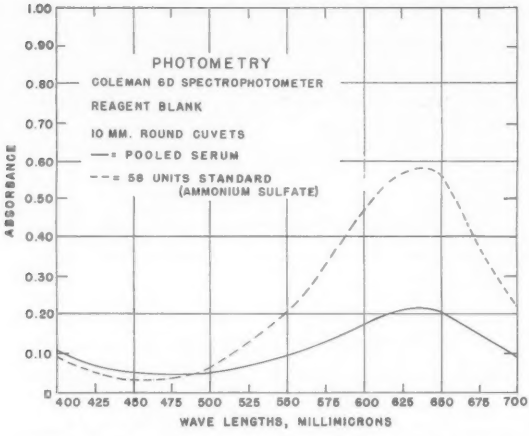


**Fig. 2.** Absorption spectra of modified Bertholet reaction with ammonia.

### pH for Adenosine Deaminase Activity

Optimal pH for adenosine deaminase activity appears to be between 6.5 and 7.2. Higher values, however, can be obtained with a buffer mixture of 4.5 parts of 2 M citric acid and 15.5 parts of 1 M $Na_2HPO_4$, as mentioned by Koehler and Benz (*1*). This latter mixture was a pH of 6.8 as opposed to a pH of 7.0–7.1 for the buffered substrate used in the proposed method.

### Storage of Serum for Blank

No difference in results was noted if serum for the blank was kept separately at room temperature for the 60-min. incubation period or if it was kept separately at 37°.

### Kinetics

It is corroborated that zero-order kinetics prevail between activity and digestion time in the range between 15 min. and 2 hr., as shown by Koehler and Benz (*1*).

### Normal Values

Twenty fasting serum samples were obtained from hospital personnel having no signs of illness. Both sexes were about equally represented in this group and ranged in age from about 20 to 55 yr. The

enzyme determinations were performed on these sera within 2 hr. after clotting at 4°.

The 95% limits for serum adenosine deaminase were 5–15 U./ml. No statistically significant difference between male and female levels was found.

## Discussion

It has been shown that an application of the Berthelot reaction (*4*), as modified by Chaney and Marbach (*3*), to the assessment of serum adenosine deaminase activity is not only possible, but highly desirable. The resulting benefits are greater simplicity with fewer manipulations, including the elimination of deproteinization. Advantage has been taken of the much greater sensitivity of the Berthelot reaction (*4*) over the Nessler reaction (*2*) to permit the development of a true micromethod. This has been achieved with slightly greater precision, greater color stability, and lower blanks than in the method of Koehler and Benz (*1*), which uses the Nessler reaction.

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# Differential Fluorometry in Catecholamine Determination: A Simplified Method of Calculation

*Shannon Brunjes and Donald Wybenga*

**A method for calculating the results of differential fluorometry in the determination of epinephrine and norepinephrine is described. A form which outlines the calculations in steps is illustrated. This simplified method is applicable to differential fluorometry and other procedures which involve solving two equations simultaneously.**

TWO SUBSTANCES with a marked difference in their excitation and fluorescence spectra can be readily measured independently. However, when the spectra of the two substances overlap, one cannot be measured independent of the other. Then the principle of differential fluorometry is utilized. Measurements are made with two different sets of excitation and fluorescence wavelengths chosen to maximize the difference between the *relative* readings of the two substances while the *absolute* readings are kept relatively large. The concentration of each substance is then determined by solving two equations simultaneously. This principle may be utilized in analyzing mixtures of substances by measuring light absorption, radioactivity, and the like, as well as fluorescence.

In the determination of epinephrine and norepinephrine, both Price and Price (*3*) and Cohen and Goldenberg (*2*) independently suggested the utilization of slight differences in fluorescent properties of the indoles formed from these compounds for their differential determination. They indicated the equations involved and stated that the two

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equations are solved simultaneously. The basic assumption is that at a given excitation and fluorescence frequency, after subtraction of the appropriate blank, the fluorescence of a mixture of two or more substances is equal to the sum of the products of the concentration of each substance present times the specific fluorescence of that substance.

"Fluorescence constants" (external standards) may be determined and utilized for all calculations. However, we have found that the fluorescence of added epinephrine and norepinephrine is variable. For accurate differentiation, especially when it is desirable to measure accurately the presence of small amounts of epinephrine in the presence of large amounts of norepinephrine, internal standards are needed with each determination. Two equations must be solved simultaneously for each determination if internal standards are used. These calculations are tedious unless done in a systematic way. Described here is a simplified method for doing these calculations.

## Method

The appropriate excitation and fluorescence wavelengths are selected utilizing curves (Fig. 1 and 2) from a spectrophotofluorometer such as the Aminco-Bowman instrument. The actual measurements

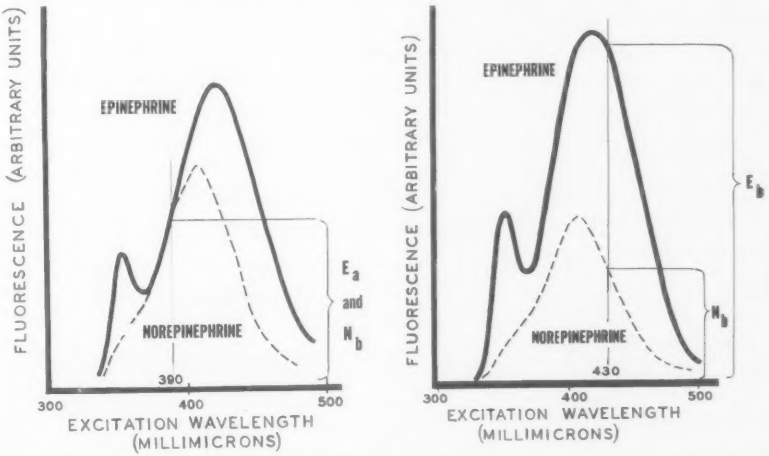


**Fig. 1** (*left*). Excitation curves of indoles formed from epinephrine and norepinephrine with fluorescence measured at a wavelength of 500 mμ. **Fig. 2** (*right*). Excitation curves of indoles formed from epinephrine and norepinephrine with fluorescence measured at a wavelength of 520 mμ.

are made with a filter fluorometer such as the Farrand using interference filters. With the "A" filters (390 mμ excitation and 500 mμ fluorescence), the indoles formed from epinephrine and norepinephrine read approximately equal. With the "B" filters (430 mμ excitation and 520 mμ fluorescence), the indole formed from epinephrine reads approximately three times as much as that from norepinephrine. Fluorescence measurements are made on four samples. One is a blank (B), one is the "unknown," one the "unknown" with added epinephrine, and one an "unknown" with added norepinephrine. The blank is subtracted from the "unknown," and the result is referred to as "U." The "unknown" is subtracted from the "unknown" with added epinephrine and again from the "unknown" with added norepinephrine, and these results are referred to as "E" and "N."

The values obtained with the "A" filters are given the subscript "a" and those obtained with the "B" set of filters, the subscript "b." $U_a$ and $U_b$ are the net readings of the "unknown" less the blank at the "A" and "B" frequencies, respectively. $E_a$, $N_a$, $E_b$, and $N_b$ represent the specific fluorescence of added epinephrine and norepinephrine at the "a" and "b" frequencies. If $x$ equals the concentration of epinephrine in the unknown solution and $y$ equals the concentration of norepinephrine in the unknown solution, the equations are: $U_a = xE_a + yN_a$, and $U_b = xE_b + yN_b$.

These equations can be readily solved for $x$ and $y$ separately, and their values summed to determine the total catecholamines. However, for convenience of calculation when these determinations are to be made repeatedly, the formula has been revised to solve for the amount of total catecholamines $(x + y)$ and for the percentage of the total catecholamines which is epinephrine $(\%x)$. The derivation of the formula for total catecholamines and for the percentage of epinephrine is as follows:

$$t = x + y$$

$$y = t - x$$

By definition:

$U_a = xE_a + yN_a$ and $U_b = xE_b + yN_b$

Solving for $x$:

$U_a = xE_a + (t - x)N_a$

$U_a = x(E_a - N_a) + tN_a$

$$x = \frac{U_a - tN_a}{E_a - N_a}$$

$U_b = xE_b + (t - x)N_b$

$U_b = x(E_b - N_b) + tN_b$

$$x = \frac{U_b - tN_b}{E_b - Nb}$$

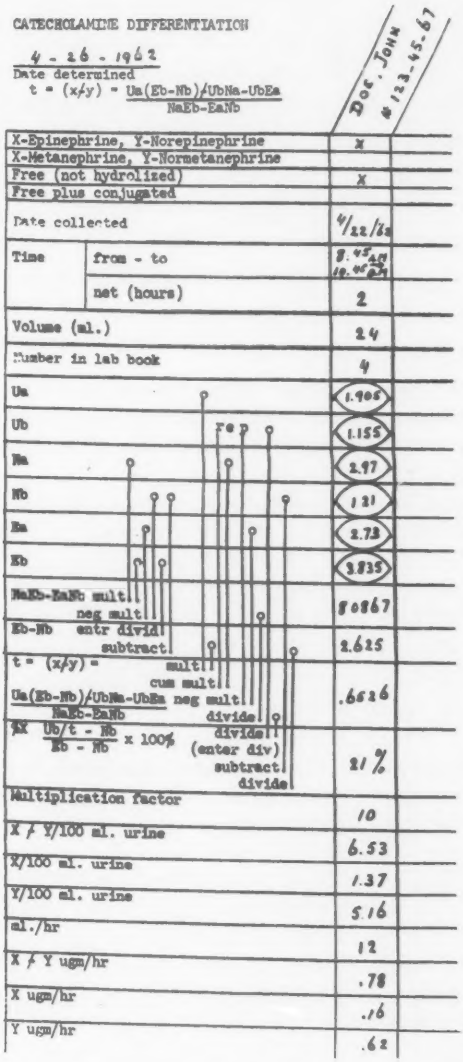


**Fig. 3.** Form used for routine calculation of urinary epinephrine and norepinephrine excretion.

$$x = x$$

$$\frac{U_a - tN_a}{E_a - N_a} = \frac{U_b - tN_b}{E_b - N_b}$$

$$U_aE_b - U_aN_b - tN_aE_b + tN_aN_b = U_bE_a - U_bN_a - tN_bE_a + tN_aN_b$$

Changing all signs and solving for $t$:

$$t(N_aE_b - E_aN_b) = U_aE_b - U_aN_b + U_bN_a - U_bE_a$$

$$t = \frac{U_a(E_b - N_b) + U_bN_a - U_bE_a}{N_aE_b - E_aN_b}$$

$$\frac{x}{x + y} = \frac{U_b - tN_b}{E_b - N_b}/t = \frac{U_b/t - N_b}{E_b - N_b}$$

$$\% \; x = \frac{U_b/t - N_b}{E_b - N_b}(100)$$

To make these calculations in a convenient and reproducible way, we use a standardized form which makes it possible for a relatively inexperienced person to calculate these values readily, using a calculator (Fig. 3). After entering the basic measured values on the form (circled), the operations to be performed on these numbers are indicated by lines indicating the type of calculations, such as multiply (mult.), enter dividend (entr. divid.), repeat (rep.), and divide. The lines extend from the indicated operation to the specific values on the table. For example, the first value to be calculated is $N_aE_b - E_aN_b$. The instruction is "mult." and lines extend to $N_a$ and $E_b$. The product of $N_a$ and $E_b$ is not cleared from the machine. Lines extend from "neg. mult." to $E_a$ and $N_b$. By using negative multiplication, this product is automatically subtracted from the preceding one. The difference between the two products is entered in the space to the right of the indicated operations. The remaining steps are carried out by following the lines from the indicated operations to the numbers to be used.

The results are in terms of a factor $t$, and the actual values of epinephrine and norepinephrine in micrograms per 100 ml. depend on the amount of urine used and the concentration of the internal standards. As these factors vary with the technic used, no constant will be given. We also use the same calculation procedure in the determination of metanephrine and normetanephrine (*1*). The specific form illustrated here was designed for use with a Friden calculator.

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# A Rabbit Serum Calcium Lowering Factor from the Pituitary

*Samuel Natelson, Joseph B. Pincus, and Gandolfo Rannazzisi*

**A factor has been shown to exist in ACTH fractions of porcine pituitaries which lowers serum calcium levels markedly when injected intramuscularly in the fasting rabbit. The lowest value is usually reached in 1.5–2.5 hr. and returns to normal in 5–6 hr.**

**It has been demonstrated that the activity of this material is independent of the ACTH activity of the preparation and is distinct from the pituitary hormones TSH, LTH, STH, or oxytocin. This factor can lower serum calcium levels in rabbits to as low as 50% of its initial value, producing tetany and convulsions.**

**It has also been demonstrated that the action of this material is not mediated through the release of l-adrenaline, l-noradrenaline, insulin, glucagon, ascorbic acid, cortisone, hydrocortisone, or corticosterone, all of which substances are without significant effect. Parathormone raises serum calcium levels slowly in the rabbit. Commercial vasopressin preparations are active in lowering serum calcium levels but not to as low a level as the ACTH preparations.**

**It has also been demonstrated that under stress the rabbit's serum calcium is spontaneously lowered to a slight degrree (10%).**

In earlier reports (*1, 2*) it was pointed out that ACTH preparations could lower serum calcium levels markedly in the rabbit, even to tetany levels. In the more recent report (*2*) it was pointed out in a footnote that we had accumulated evidence to indicate that this effect was not due to ACTH but to some other substance in the ACTH preparations. The purpose of this report is to marshal that evidence.

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## Procedure

The method used in testing the different substances has been described in the previous report (*2*). The materials tested, their source, and their effect are listed in Tables 1 and 2. The animals were fasted for 24 hr., but allowed access to water. A fasting blood specimen was taken and subsequent hourly specimens, after intramuscular injection of the sample, for a 5-hr. period. The results reported in the tables represent the greatest percentage drop from fasting. This usually occurs at the second hour and returns to normal at the end of 5–6 hr. in most cases. To eliminate sensitization effects no rabbit was used more than once. Calcium estimation was done by X-ray spectrometry (*3*).

## Discussion

Table 1 summarizes the data obtained by studying different preparations of ACTH and comparing their ACTH activity (ascorbic acid

**Table 1.** Effect of ACTH Preparations on Serum Calcium Levels in Fasting Rabbits

| *Polypeptide* | *Source* | *ACTH activity (U./mg.)* | *Dosage (μg./kg.)* | *% Fall serum Ca (mean)* | *No. rabbits* | *Comments* |
|---|---|---|---|---|---|---|
| ACTH Std. La-1a | Armour | 1 | 300 | 22 | 10 | Reported in Natelson *et al.* (*1*) |
| ACTH Lot No. 128-105 | Armour | 1 | 300 | 32 | 10 | |
| ACTH Lot No. I-101 | Armour | 25 | 80 | 35 | 10 | Oxycellulose purified corticotropin A; see White (*8*) |
| ACTH Lot No. 216-177-3 | Armour | 25 | 80 | 22 | 10 | |
| ACTH, $NaHCO_3$ insoluble | Armour | 30 | 100 | 27 | 7 | $NaHCO_3$ insoluble fraction before fractionating on the oxycellulose column. |
| Fraction $A_1$ No. 907-109-$A_1$ | Armour | 1 | 100 | 29 | 6 | 1st peak off the oxycellulose column. |
| ACTH, CMC Frac. No. 1193-017-021 | Armour | 0 | 150 | 25 | 7 | Fraction from a carboxy methyl cellulose column. |
| | | *U./ml.* | | | | |
| ACTH, injectable | Wilson | 40 | 2 U./kg. | 33 | 8 | Commercially available ACTH; Wt. unknown and variable. |
| ACTH, injectable | Armour | 40 | 2 U./kg. | 28 | 6 | Studied at N.I.H. by Dr. R. C. Likins. |

depletion test in rats) with their activity in lowering serum calcium levels in the rabbit.

It will be noted that one fraction with no ACTH activity is quite active in lowering serum calcium levels. However, one fraction with an ACTH activity of 1 U./mg. has an activity of the order of another with 30 U./mg. with respect to the lowering of serum calcium levels. It will be also noted from Table 2 that other hormone fractions obtained from the anterior pituitary have little or no effect.

None of these pituitary fractions for which data are shown in Tables 1 and 2 are homogeneous, electrophoretically. At least three to four major components could be observed in each case with paper electrophoresis with a tris-citrate-borate buffer at *p*H 8.0 (*4*).

The data of Table 2 indicate that other polypeptides such as insulin, glucagon, and parathormone are without significant effect in lowering serum calcium levels. Other physiologically active substances such as l-adrenaline, l-noradrenaline, corticosterone, cortisone, or ascorbic acid are either without effect or of such minimal effect that one cannot say that the calcium lowering factor acts by stimulating their secretion.

**Table 2.** Effect of Various Hormones and Related Substances, other than ACTH Preparations, on Serum Calcium Levels in the Fasting Rabbit

| *Substance* | *Source* | *Dosage* | *Max. % change in serum Ca level (mean)* | *No. of rabbits* |
|---|---|---|---|---|
| TSH, No. 216-177-5 (bovine thyrotropin) | Armour | 150 | + 4 | 6 |
| STH No. D-728-076 (bovine somatotrophin) | Armour | 150 | −10 | 6 |
| LTH No. D-14083-2B (ovine luteotropin) (purified) | Armour | 150 | −10 | 6 |
| Oxytocin (Pitocin, 10 U./ml.) | Parke, Davis | 2 U./kg. | none | 6 |
| Vasopressin (Pitressin, 20 U./ml.) | Parke, Davis | 2 U./kg. | −19.4 | 6 |
| Glucagon (eryst.) | Lilly | 140 μg./kg. | −10 | 7 |
| Insulin, U.S.P. regular | Lilly | 0.25 U./kg. | − 6 | 5 |
| Parathormone | Lilly | 1000 U./kg. | + 5 | 6 |
| l-Adrenaline | Eastman | 100 μg./kg. | − 8 | 10 |
| Hydrocortisone | Mann | 20 mg./kg. | none | 7 |
| l-Noradrenaline | Mann | 100 μg./kg. | − 5 | 7 |
| Corticosterone | Mann | 20 mg./kg. | none | 6 |
| Ascorbic acid | Eastman | 50 mg./kg. | none | 4 |
| No stimulation | — | 2 ml. | none | 6 |
| Saline injection with stimulation | — | 2 ml. | −10 | 6 |

Of special interest is the effect of the hormones from the posterior pituitary. Oxytocin is without effect, but commercial vasopressin has a definite effect in lowering serum calcium levels in the rabbit. When a purified preparation of vasopressin was used (vasopressin, 60 U./mg.*) at the 100 μg./kg. level (6 U./kg.), a mean calcium depression of 23% was observed in 4 rabbits. None of these rabbits was in as severe distress as those treated with the preparations of Table 1.

Vasopressin and a substance associated with vasopressin, but not completely identified, stimulate the anterior pituitary to secrete corticotropin (*5, 6*). The question then arises as to whether vasopressin has this action per se, or whether it acts to stimulate the anterior pituitary, its action being thus indirect. Another possibility is that the same factor occurs as an impurity in the ACTH preparations and the vasopression preparations. While vasopressin increases calcium excretion in the urine it has no effect on serum calcium levels in dogs (*7*). This problem is being studied with hypophysectomized animals to resolve this question.

That a mechanism for lowering serum calcium levels acutely exists in the intact rabbit can be seen from Table 2. In successive blood samples taken from a rabbit untreated or treated with saline but not stimulated, the serum calcium level is constant. If the rabbit is vigorously stimulated by being frightened, or if a drop of xylene is dropped in his eye (this is very irritating to the rabbit) a significant drop in serum calcium levels will occur rapidly and then return to normal. Thus, in a typical experiment, stimulation of a rabbit produced a 10% drop in serum calcium levels which persisted for 30 min. after stimulation and returned to normal at the end of an hour. This could be done repeatedly in the same rabbit. Thus one may say that in acute stress a prompt lowering of serum calcium can be produced in rabbits.

For the above reason, lowering of serum calcium levels of the order of 10% for the intact rabbit, cannot be considered significant. Thus in Tables 1 and 2, only the ACTH and vasopressin preparations can be considered to exhibit activity.

Attempts to assay the various preparations relative to each other were unsuccessful, as illustrated in Table 3. These three preparations seem to be approximately of the same activity at the 100 μg./kg. range but are markedly different at the 150 μg./kg. range. Further, variation from rabbit to rabbit on the same material is too wide to permit

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**Table 3.** Effect of Increasing Dosage of Active Preparations on Serum Calcium Levels in the Fasting Rabbit*

| *Dosage (μg./kg.)* | *ACTH $NaHCO_3$ insol. fraction* | *ACTH No. 907-109-A* | *ACTH No. 216-177-3* |
|---|---|---|---|
| 20 | 7.9 | 11.1 | 7.5 |
| 50 | 13.0 | 18.0 | 13.6 |
| 100 | 27.0 | 29.0 | 24.0 |
| 150 | 36.0 | 49.0 | 39.0 |

*Values are the mean percentage drop from the fasting level, for 3 rabbits.

of accuracy of assay unless a large number of rabbits is used as may be seen in Table 1 in the paper by Natelson *et al.* (*2*). For this purpose better results may be obtained in hypophysectomized rabbits; this is being investigated.

From the material above it is apparent that a factor which lowers serum calcium levels in rabbits exists in extracts of the pituitary, apparently distinct from ACTH, TSH, LTH, STH, and oxytocin. This factor is found associated with the ACTH fractions of the pituitary. Some activity is observed in vasopressin preparations. However, severe lowering of calcium levels to tetany levels was observed only with the ACTH preparations. Other polypeptides and nonprotein hormones listed in Table 2 are without significant effect.

For the reasons stated above we feel that we have demonstrated that such a factor exists and is probably distinct from the known hormones. Whether it is of biologic significance itself or acts by indirect stimulation of some other mechanism in the rabbit will only be decided by further study.

At present we are attempting to isolate this material and study its effect in adrenalectomized and hypophysectomized animals.

## Addendum

Since this report was submitted a highly potent preparation that lowers rabbit serum Ca levels by 45% at the 50-μg./kilo level has been prepared from *human* pituitaries. Acetone-dried powder prepared from 40 human pituitaries (10 gm.) was extracted with 0.1 N HCl in 70% acetone. After filtering, 700 mg. of powder was obtained by precipitating with acetone to bring it to 95%. The powder was dissolved in 200 ml. 0.1 N acetic acid containing 75 mg. Na acetate and adsorbed to oxycellulose. Elution from oxycellulose with 0.1 N HCl yielded, on precipitation with acetone, 10 mg. of the highly active crystalline prep-

aration. This same technique applied to animal anterior pituitary powders has produced highly active preparations suitable for further fractionation by electrophoresis and column chromatography.

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the Clinical Chemist

## Clinical Chemistry Sessions, at 130th Annual AAAS Meeting Sheraton-Cleveland Hotel, Cleveland, Ohio

### Dec. 26-31, 1963

### Preliminary Outline

**Dec. 26**

2:00 p.m.: Session I—General session

**Dec. 27**

9:00 a.m.: Session II—Symposium on Chromatography

Applicability of Chromatography to Clinical Problems, Dr. Erich Heftmann, Laboratory of Chemistry, National Institute of Arthritis and Metabolic Diseases, National Institutes of Health, Bethesda, Md.

2:00 p.m.: Session III—Symposium on Microchemistry

The Use of Electronics in Analytical Chemistry, Dr. Harry L. Pardue, Department of Chemistry, Purdue University, W. Lafayette, Indiana.

Further information may be obtained from Willard R. Faulkner, Ph.D., Program Chairman, The Cleveland Clinic Foundation, Cleveland 6, Ohio.

## New Translation and Abstract Service

The Extramural Program of the National Library of Medicine has awarded four contracts for the translation and abstracting of the foreign biochemical literature and for the preparation of a directory of Soviet research institutions.

The contracts, made under NLM's responsibility for helping to improve the international exchange of biomedical information, are designed to speed and broaden the communication of significant foreign literature in medicine and biology to the American scientific community.

Work under the contracts involves nearly all disciplines of medicine and biology and Russian, Chinese, Japanese, and Slavic languages.

The contractors and outlines of the work to be performed follow:

(1) Biological Abstracts, Inc.,

Philadelphia, Pa., will translate and/or prepare abstracts in the basic biological and medical fields from Soviet biomedical literature. These abstracts will be published in the semimonthly journal *Biological Abstracts,* and will be integrated by discipline with similar world-wide work appearing in the journal.

(2) Fordham University, Bronx, N. Y. will compile a "Directory of Medical and Biological Research Institutions in the USSR." The Directory will include a description of the organizational structure of Soviet scientific activity in medical and allied fields, and a listing of schools, libraries, societies, and pertinent publications.

(3) The Excerpta Medica Foundation, Amsterdam, Netherlands, will translate and/or prepare abstracts of Soviet and Japanese biomedical literature to be published in the appropriate subject sections of *Excerpta Medica.* The project will provide a comprehensive review of development and progress in Soviet and Japanese medical research and clinical practice, together with reports on Soviet and Japanese progress in all fields of medicine.

(4) The Federation of American Societies for Experimental Biology, Inc., Bethesda, Md., will provide selective translation and publication of Soviet and other foreign biomedical research information. This project will provide dissemination of significant foreign research results to the scientific community through the "Translation Supplement" to *Federation Proceedings,* the FASEB journal.

The Extramural Program also will continue the publication of the *Bibliography of Medical Translations,* a listing of selected translations in the biomedical fields compiled by the Office of Technical Services from its publication *Technical Translations,* as a quarterly current-awareness publication.

Additional information regarding these projects may be obtained from: Science Translation Program, NLM, Bethesda 14, Md.

## Minutes of Executive Committee Meeting

The meeting was called to order at 6:30 p.m., Aug. 20, 1963. Present were R. J. Henry (President), J. Sendroy (President-Elect), M. Reiner (Past President), R. L. Dryer (Secretary), M. Kaser (Treasurer), W. B. Mason (Ethics Committee), W. R. Faulkner (Public Relations Committee), W. T. Caraway (Membership Committee), A. Hainline (Past Chairman, Membership Committee), O. H. Gaebler (AAAS Delegate), and H. D. Appleton (Editorial Board).

Serving as official delegates to the Committee were: E. W. Bermes (Chicago), M. E. Ryland (Philadelphia), M. Knowlton (Capital), R. P. McDonald (Michigan), B. J. Katchman (Ohio Valley), Alex Kaplan (Puget Sound), J. I. Routh (Midwest), M. Friedman (Metropolitan New York), W. Golden (Connecticut, serving as

alternate for N. California), M. Murray (Upstate New York), J. R. Elliott (Texas), D. S. Remp (At Large), A. G. Ware (S. California), R. S. Melville (At Large), G. T. Lewis (Florida), A. Hainline (Cleveland), K. Dubowski (At Large), E. W. Rice (Pittsburgh), and G. Bowers (Connecticut).

Following the call to order and the introduction of delegates, the first item of business was a review and discussion of the financial report and the budget by the Treasurer. These items had been presented and approved at the interim meeting last April. The present review was to clear up some misunderstandings which had apparently grown out of the brevity of the April report. It was pointed out that the major differences between the present budget and those of the past was found in just three items—the expenses of the Editorial Board, the Secretary's Office, and the Treasurer's Office—in that order of magnitude. To cut the publication delay of the journal to a reasonable value of 4 to 6 months it has become necessary to change to a monthly publication period. The increased work load which this change entails will require a considerable increase in the operating expenses of the editorial office; to compensate the Managing Editor for what amounts to a half-time responsibility, a stipend of $4000 per year was approved. The fiscal year of the Association runs from July 1 of each year, while the journal year begins January 1. It was pointed out that the lead time necessary to edit and compose the journal was such that provision for these expenditures would be required before any revenue from increased advertising or circulation would be received. Still, the advance to the account of the Editorial Board, which appears as a deficit in the current budget, should be more than offset by 1965 as the increased revenue is collected.

The Secretary pointed out that the expenses levied against his office include all printing, postage meter rental and postage costs, all telephone tolls, a modest allowance for stenographic and clerical assistance, and a personal allowance for travel. For the past 2 years these expenses have totaled about 70% of the sum budgeted. He mentioned that it has been possible for him to use office facilities furnished by his hospital in connection with the performance of his duties, but that some time in the very near future these facilities will no longer be available. This will require a rather different set of circumstances. Three alternatives present themselves. First, together with the required secretarial assistance, commercial office space could be rented and furnished. It was estimated that this might cost $3200 for the coming year. Second, the office could be moved to the home of the Secretary, for which he requested compensation of $400 per year. With the same figure for clerical assistance, this would involve the expenditure of $2400 per year. The third alternative was to transfer the office of the Secretary to some other person better able by professional con-

nection to support it. The Finance Committee had approved the second alternative. Since the exact date of the changes anticipated by the Secretary cannot be established, it is quite probable that the actual expenses for the current fiscal year will not be as great as the amount budgeted.

The Treasurer's office expenses will also rise due to the continued expense of professional audit and accounting, the need for more detailed tax records, and the increased level of general Association expenditures. The Association ledger was displayed for the benefit of the members present.

The above explanations of fiscal issues were accepted by the Committee. In the discussion which followed some objections were raised concerning the disproportionate increase in operating expenses as compared to the growth in membership. The Treasurer rebutted these objections with the comment that the net worth of the Association has nearly doubled in the same period of time as in formulating the objections. It was agreed that detailed financial statements and budgets should be sent to each member, and that annual statements on dues should show the cost of journal subscriptions. It was so ordered.

In related matters of financial policy, the Committee agreed to increase the sum budgeted for meetings, held as an affiliate body of the AAAS, from $100 to $300. A resolution submitted by the Puget Sound Section to the effect that dues for affiliate members be lowered by eliminating the required journal subscription was formally rejected. It was instead moved and passed that dues for affiliate and associate members be the same, and that journal subscriptions be required for all classes of membership. In accordance with constitutional requirements, the Committee formally approved the recommendation to the membership that dues for 1964 not be increased by more than the sum of $2.00 to cover the cost of the enlarged journal (12 issues instead of 6), so that the dues structure will be as follows:

| | *Associates and Affiliates* | *Members* | *Fellows* |
|---|---|---|---|
| Journal | $ 8.00 | $ 8.00 | $ 8.00 |
| Dues | 5.50 | 9.00 | 11.50 |
| Totals | $13.50 | $17.00 | $19.50 |

The motion in favor of the above recommendation was carried, but not unanimously.

Prior to the meeting, a proposed text of a brochure to be entitled "Careers in Clinical Chemistry" had been circulated among the members of the Committee. This was brought up for discussion. It was agreed that a somewhat more elaborate and detailed text would be advantageous, and the problem was referred back to committee for elaboration. The hope was expressed that this would be considered again at the next meeting.

The Committee on Constitutional Revision had also circulated detailed proposals for changes which they considered suitable. Discussion of these proposals revealed considerable disagreement, especially those which touched upon the oganization of the

executive body. The chair therefore acted to return the matter to committee. In so doing, the members of the Committee were urged to present any specific objections or suggestions in writing. The matter of changing the membership of the constitutional review committee was taken under advisement by the chair.

The retiring chairman of the Membership Committee reported that as of the present date, 37 affiliate members have been elected to the Association.

There was no report from the Standards and Controls Committee.

The Committee on Standard Methods reported that Volume 4 will be ready in October 1963, and that work is already well underway on the preparation of Volume 5.

The Ethics Committee indicated that they had faced only one problem on ethics in the past year, and stated that this had been easily solved.

The Committee on Legislation has had a busy year, with much interest and considerable activity in matters of legislation in several of the state legislatures. The Committee has worked out what it considers a proper stand; this had been approved by a past action of this Committee. While it had been subject to some criticism by individual members, the present position was felt to be the safest possible. The Committee asked that some means of providing funds for travel or necessary correspondence be provided for emergency use. This was approved.

The Education Committee had no report other than to announce that the first NIH Training Grant in Clinical Chemistry had been awarded to Dr. Alex Kaplan, of the Puget Sound Section. Questions about the training grant program should be addressed to the Chairman of the Education Committee, Dr. Martin Rubin, Georgetown University, Washington, D. C.

The Employment and Personnel Service has been taken over by Adrian Hainline who announced that an increased program is being contemplated; this will include the possibility of box ads in the "Clinical Chemist" and the further possibility of arranging formal interviews during annual meetings.

The Fellowship Committee announced that the first grant of a graduate fellowship in clinical chemistry had been made to Mr. Alex Slonicki of Georgetown University. This fellowship is made possible by a gift from the Warner-Chilcott Company, supplemented by Association funds.

The delegate to AAAS, Dr. Gaebler, announced that he has asked Dr. Willard Faulkner to preside at the annual meeting of the AAAS, which will be held in December at Cleveland.

The delegate to the Intersociety Committee, Dr. R. P. McDonald, reviewed the history of the liaison between it and this Association. He urged that the connection be strengthened since it now seems that other alternatives are not forthcoming to succeed the Intersociety Committee's efforts. His request for travel grants was accepted.

The Screening Committee for the

Ames Award asked for advice in the matter of selecting award nominees. The matters of nationality, age, prominence in service vs. prominence in research were mentioned as possible criteria. It was moved that on the record of accomplishment, the Committee be given the widest powers of discretion in these matters. The motion was approved. To avoid possible embarrassmen ·ver nomination of members of the Committee, it was agreed that two additional members who need not both be award winners of the past should be appointed. The chair will proceed in accordance with this advice.

The Capital Section had presented a revised code of bylaws for approval. In the opinion of the Secretary these were in order, and on past precedent he moved that they be accepted. Following brief discussion, it was so moved. The issue of local section dues was raised in the general discussion of Section bylaws. Provided the assessment carries no punitive stipulation, it was agreed that local sections may, at their discretion, collect funds from their members.

The Secretary announced that the recent election for President-Elect was clear and in favor of Dr. J. Sendroy. The election for Delegate-At-Large to this Committee was clear and in favor of Dr. R. S. Melville. Election of the Nominating Committee had resulted in a four-way tie for two places, and the four contending candidates were paired from two local sections. Election of the last two members was therefore made by the Committee, which acted to break the tie.

The position of Chairman of the Board of Editors of CLINICAL CHEMISTRY has been vacant since the creation of the post of Managing Editor. Upon recommendation of the Editorial Board, a slate of names was considered. By secret ballot Dr. J. Sendroy was elected to serve a 5-year term as Chairman of the Editorial Board.

The Secretary formally placed before the Committee invitations from the City of New York, the City of Philadelphia and the Philadelphia Section to serve as hosts for the earliest possible annual meeting of the Association. It was pointed out that a past action of this Committee limited the scheduling of meetings more than 3 years in the future. It was felt by several of those now involved in the planning of forthcoming meetings that such a limitation did not always allow sufficient time to schedule desirable hotels and speakers, and time to perform the many necessary functions of meeting organization. With this in mind, the limitation was formally changed to 4 years in advance of the calendar. Following this action, the invitation of the Philadelphia Section was accepted for 1967, and the section delegate was so informed.

On behalf of the Metropolitan New York Section, a resolution was introduced pointing out that several of the smaller sections might not have adequate programs due to lack of

funds. The resolution therefore asked that the sum of $100 be given to each section in addition to the annual membership rebate. A poll of several delegates of small sections revealed that they were not in serious financial straits, and in the lack of apparent sentiment for such an appropriation, the motion, which would have implemented this resolution, died for lack of support.

A warm vote of thanks was moved to the Michigan Section and to our Canadian colleagues who worked so efficiently to organize the Fifth International Congress on Clinical Chemistry.

The meeting then adjourned.

## Minutes of the Membership Meeting

The stated annual meeting of the membership was called to order at 4:30 p.m., Aug. 21, 1963.

The first item of business was the presentation of the Scientific Products Foundation Award, a glass bowl suitably inscribed, to Dr. Miriam Reiner, the outgoing President of the Association.

The Secretary announced that registration figures available at the time indicated that Congress registrants came from 29 countries, that a total of 848 registrants had been in attendance and at least 646 were full members.

The President called for presentation of the minutes of the Executive Committee meeting which were read by the Secretary. The minutes were accepted and approved without comment. In discussion of the actions represented by the minutes, as read, it was asked by Dr. Friedman that the budget be returned to the Finance Committee for review of a more equitable funding of the activities of some committees, and for general reconsideration in view of membership growth rates. He urged that some means be adopted to indicate to the membership that dues were too low. The Treasurer defended the budget stressing the spirit of economy in which it had been formulated. She added that the Finance Committee tended to underestimate income and overestimate expenses, as the past record shows. With the advent of new sections, the separation of the officers, and increased Association activity on several fronts, expenses are bound to rise. She repeated the hope that no cash deficit will result this fiscal year in spite of several contingent items which appear in the budget. The Secretary reviewed several proposals relative to his office and indicated that in spite of official approval of a proposal to move the office to his home, some adverse comments had been heard as to the propriety of such a move. In the interests of harmony, he offered to consider the matter on a trial basis without compensation, reserving the right to withdraw.

The chair called for consideration of annual dues. It was moved and seconded that the dues for the fiscal year 1964 be raised by the sum of $2.00 to cover the increased cost of journal subscriptions. Some discus-

sion indicated a minority in favor of a greater increase. On a voice vote, the recommendations of the Executive Committee was overwhelmingly accepted.

Dr. Saifer pointed out that the next International Congress of Biochemistry will be held in New York City, and asked if any formal arrangements had been made for participation by the Association. The Secretary indicated that there had not, but stated he would attempt to arrange some degree of affiliation.

For the membership, the President reviewed the plans for the next four meetings. Insofar as it has been established, the calendar of meetings of the Association reads as follows:

1964—Boston, Aug. 17-20

1965—Chicago, Aug. 30–Sept. 2

1966—Florida

1967—Philadelphia

Dr. Faulkner briefly discussed the forthcoming joint meeting with the AAAS, and urged that those desiring to submit papers do so as early as possible in September.

The meeting was then adjourned.

# Donald D. Van Slyke:

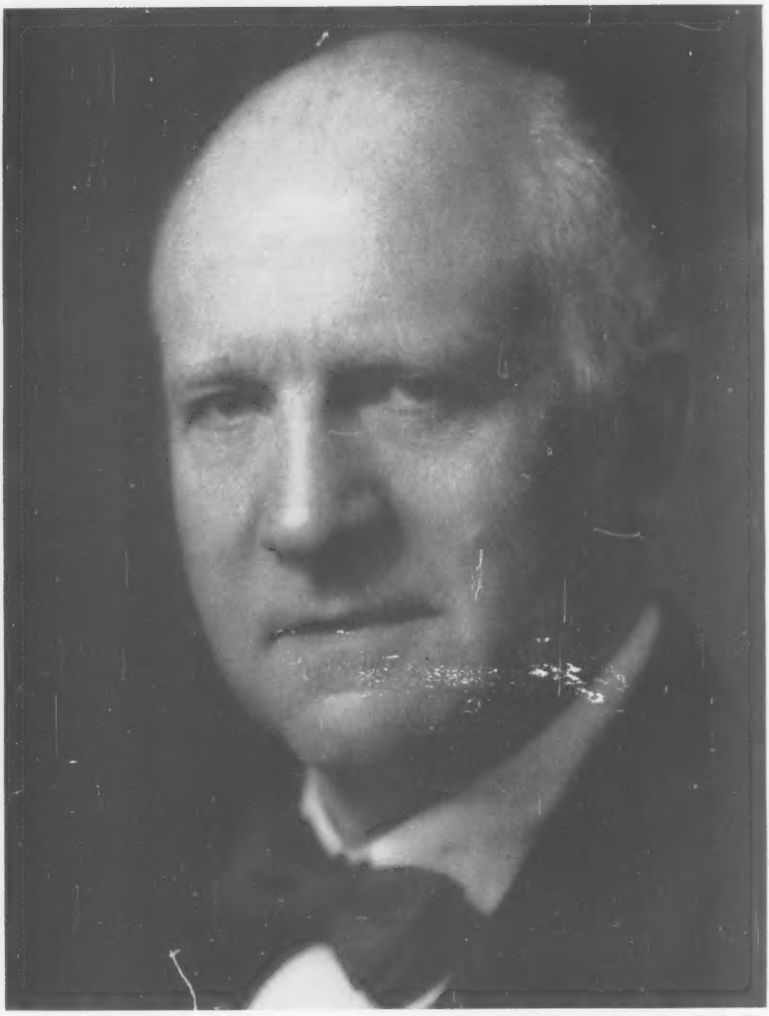
## On His 80th Year

DONALD D. VAN SLYKE celebrated this past March twenty-ninth his eightieth birthday and his fifty-seventh year of constant scientific accomplishment. The body of knowledge that he so actively built over this period of years can be said to have put *science* into the *art* of medicine.

Dr. Van Slyke is a clinical chemist, and one whose contributions have stood the test of time. He has actively shown the place of the chemist in the modern medical approach. In 1925 he was rated by the American Chemical Society as one of the 39 most eminent American chemists. In 1962 he received the Scientific Achievement Award from the American Medical Association. His contributions to the understanding of biochemical processes and their assay date to 1906 and are constant reminders of how pure science has advanced the knowledge of life's mechanisms.

Van Slyke was born in Pike, N. Y., Mar. 29, 1883. He received his A.B. from the University of Michigan in 1905 and his doctorate in 1907. He has been awarded honorary degrees of Doctor of Science by the University of Michigan; Yale, Northwestern, and London Universities; and the University of Chicago. In 1938 he received an honorary Doctor of Medicine from the University of Oslo and more recently received the same degree from the University of Amsterdam.

He is an emeritus member of the Rockefeller Institute, having been associated with that group since 1907. Since 1948 he has continued his work at the Brookhaven National Laboratories at Upton, New York. His scientific achievements encompass six major areas of investigation: (1) proteins, amino acids, enzymes; (2) quantitative clinical methods; (3) acidosis, acid-base balance; (4) gas and electrolyte equilibria; (5) renal function and nephritis; and (6) isotopy. His students and associates have gone on to bring his type of scientific thinking, curiosity, and accomplishment into other related fields. All confess the influence of their association with him, and in many instances they still come for advice and guidance.



Courtesy Rockefeller Institute, New York City

**Donald D. Van Slyke, Ph.D.**

The debt that the clinical chemist owes to Dr. Van Slyke is immeasurable, not only for the excellence and practicability of most useful methodology, but also for the underlying philosophy that these are tools which should be used not just in the detection and treatment of disease but in wider applications to fundamental studies of life processes.

In his eightieth year Dr. Donald D. Van Slyke has once again received honors. The Brookhaven National Laboratory held a special 2-day meeting and the American Microchemical Society held a symposium in his honor. We felt that the dedication of an issue of our Journal as a *Festschrift* would be our salute to the Association's eminent member on this occasion, in appreciation of his generosity through the years in giving us the benefit of his knowledge, experience, and encouragement. We all wish him many more happy and fruitful years.

—The American Association of Clinical Chemists

## DONALD D. VAN SLYKE

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# One of God's Children; One of Science's Best

*Irvine H. Page*

IT IS SAID of Donald Van Slyke that he is a good man. This expresses simply and eloquently, what most people think of Van. In fact, sometimes he is good to the point of forgetting that there is a bad side to some human affairs. But let's be more specific. Van has stood for something in science quite other than his contributions to knowledge, and quite other than his general goodness as a human being.

Humility is a rare thing in reality, and yet one of the most commonly proclaimed virtues. Who does not with mock humility deny his worthiness? Who does not give credit to all and sundry who have "made my work possible"? Who does not promise to carry out, "this great responsibility with what little talent I have"? Who does not "accept in all humility this great honor you have bestowed on me"? Truly, it is rare to find genuine humility, for the simple reason most of us don't feel it. We mostly use it as a cover-up to keep our pride from showing through too patently. During seven years' working with Van I saw true humility and true pride. They were both honest.

Integrity is not much used these days as a characterization. We prefer to speak of "good old-fashioned honesty." Honesty may be old-fashioned, but probably is not more so today than it ever was. But integrity is one of the pillars of science and I should think it rare that most scientists do not know this and behave accordingly. The problem is that integrity gets shaved around the edges too closely. If I were to pick a single cause for this parsimonious use of integrity, it would be that scientists are a little too much in a hurry to claim the credit of accomplishment for themselves and their institutions. Just one example —the fourflush with which investigators head off possible future competition by expansive verbal claims. When in voice, a good fourflusher can pretty much blanket the frontiers of a problem with implications and claims of discovery. In contrast, the full use and meaning of the word integrity are in the man called "Van Slyke." He knows no ruse,

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no guise, no dissemblance. Such patent integrity all too often disconcerts us ordinary mortals and this is because we often lack the more exacting variety.

Persistence seems a prosaic virtue. Many people are persistent because they are too stupid or stubborn to be otherwise. A wet dog also is persistent in his friendliness. But the persistence of a scientist who continues in his research until he has built a body of knowledge of which he may be proud is another thing. All too often we are afflicted with the occupational disease known as "scientific opportunism." With each new announcement of another's work we follow the new piper, even though he may be quite "pied." Too often we recognize in our own work the pattern characterizing Brownian movement; it starts nowhere and goes nowhere, yet keeps up endlessly frenetic agitation. Heat makes it more active and that is why we speak of a "hot subject."

Research, to Van, has never been a luxury or a way to the top. It has been, and is, something he deeply loves. It is truly a way of life with him. Persistence has come naturally because of his motives. He has known the beauty of the body of knowledge which results from persistence. Does not the "Van Slyke machine" typify this? Its persistent use as a tool of analysis has done as much as any other single force to unify the clinical laboratory and bedside medicine. Who more accurately and persistently followed the "natural history" of nephritis and nephrosis? That, indeed, required persistence. No, Van Slyke is not a "scientific opportunist"; he has not lived a life of gamesmanship. Because he loved to do research, Van has been patient and persistent because he is so and because he knows these qualities to be virtues in the conduct of research. This kind of broad-scale work does not win Nobel prizes but it builds a body of knowledge for which mankind is always the better.

Van grew up in an era when the "politics" of science neither had any great impact nor was considered altogether respectable. This was the day when there were two operational systems from which authority stemmed. The one, "papa knows best," was a residue of the German "Geheimrat system" and the other, the English "flowering of knighthood" or "old boy" club. In this country at that time we had a bastard mixture of both. We hardly knew what we had and, curiously, we are only now beginning to wonder because of periodic and rather serious eruptions in some of our better known clubs and institutes.

We are faced with an odd melange of democracy and autocracy.

There are scientific organizations so elite they have nearly guaranteed their own demise, while democracy reaches its apogee in organizations whose requirement of excellence is that you have the income-tax-deductible dues. Often a cruise is thrown in. Perhaps it is all summed up in Groucho Marx's "I'm not going to pay good money to join a club that lets in people like me."

And what has this to do with Van? Just this: Van belongs to every "status society" of which I am aware and to the "nonstatus ones" as well. But he either doesn't know it or ignores this intellectual segregation. This man doesn't even think in these terms. To him, people and science are good, and you need look no further. People know he is right, but the game of politics for them is still too strong. While knowing he is right they continue the game of you-scratch-my-back-and-I'll-scratch-yours.

I can't, however, look on politics in science as all bad. Politics in the sense that it is skill in the science and art of government deserves no sneers. It is at the bottom of how we scientists are going to get along with each other and the ambient world. And from the way things are going at the moment, I suggest we need more, not less, of decent politics.

Van was, as I have said, brought up in the age of politics for the very few. But see what has happened in the past 20 years. We are up to a 5-billion-dollar budget and I believe it is true that 90 per cent of all scientists are alive today who ever lived. We are now big business and have assumed an enormous responsibility both for the defense of country and for a part in leading its thinking as well. Who but scientists have the answers now? Haven't the Nobel prize winners displaced the oracles of the business world of forty years ago? And I may say I think it is an improvement, though some do not agree.

So politics in the good sense has become an essential ingredient of science. Further, I think we could agree that most of us have only the slightest idea of how to deal with these problems in a way to do credit to us as scientists. There is no organization or society to deal with them for us. There are few ways for the individual scientist's voice to be heard. All we have today are bits and pieces of what must some day be put together into a structure which can handle the political and managerial problems of science in some orderly and reasonable fashion. I'm not sure Van would understand this altogether. The Rockefeller Institute under Dr. Flexner was not an environment to encourage broad participation in the political affairs of science, and Van so

epitomized the dedicated research worker he probably would not have been interested anyway. I think this shows that no man can be everything to all men.

A lesson to be drawn from Van's career is that research can be a complete and rewarding experience. It does not necessarily require teaching or participation in politics to be satisfying. This does not mean that the latter are not desirable; they *are* only if the investigators want it that way. There should be plenty of room and opportunity for several ways of life for a research worker. We must, as scientists, beware of lying down on Procrustes' bed. We should be allowed within reason and prudence to make our own beds if we have to lie on them.

There is so much in a man like Van that a younger generation of scientists should heed. I must draw a line, but not before mentioning one other quality. Van is a tennis player and a good one. He has not quit for fear his heart would fail before his brain. The interesting thing about his game is that it exhibits almost none of the virtues I have extolled as the best in a scientist. No, perhaps one, and that is persistence. Van is human enough not to want to lose.

Perhaps I have dwelt on virtues to the point that Van Slyke no longer appears as one of us. In terms of accomplishment he isn't one of us; he has just done too much. But as a human being they come no better, with such a nice mixture of gentle vice and virtue. Surely no one, including Van, would have it otherwise.

# Microdetermination of Chloride in Blood Plasma and Cells, by Spectrophotometric Analysis Using Solid Silver Iodate

*F. Lee Rodkey and Julius Sendroy, Jr.*

**As an extension of the silver iodate method of Sendroy, a convenient, reproducible, and sensitive method for analysis of chloride in 0.02-ml. samples of serum, whole blood, or red cells is described. The procedure involves (1) preparation of a protein-free filtrate of the sample, (2) $AgIO_3$ reaction with the filtrate, and (3) spectrophotometric analysis of iodine as the quantitative measure of the amount of iodate exchanged for chloride. Conditions important for each of these steps have been investigated. A precision of ± 0.85 (S.D.) mM Cl per liter has been found for this method, the results of which are within 1% of those obtained by the macro nitric acid digestion method of Van Slyke.**

THE DETERMINATION of chloride in solution, based on its reaction with solid silver iodate, was first described by Sendroy (*2*) with specific applications to a system of analysis of blood, plasma and urine (*3–5*).* In these methods, in which sample size ranged from 1.0 to 0.02 ml., iodate liberated from silver iodate by reaction with soluble chloride was analyzed by gasometric, titrimetric, and colorimetric proce-

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Samples of human serum and blood used in testing the new method described were obtained from the Naval Medical School, National Naval Medical Center, courtesy of LCDR. Thomas E. Wheeler (MSC), USN.

The opinions or assertions contained herein are the private ones of the authors and are not to be construed as official or reflecting the views of the Navy Department or the naval service at large.

*It is a pleasure for me to recollect and to note here that the methods cited were developed in Dr. Donald D. Van Slyke's laboratory at the Hospital of the Rockefeller Institute as a result of his suggestion that the principle of differential solubility might be utilized for chloride analysis.—J. S., Jr.

dures. Since the basic theoretical considerations were adequately presented in the foregoing papers, they will not be repeated here. It was found (*3*) that removal of protein from plasma (or serum) or urine was not necessary for the analysis of iodate by the gasometric or titrimetric procedure. However, colorimetric analysis required the deproteinization of such samples. Moreover, all analyses of whole blood, regardless of the method of iodate measurement, were likewise done on protein-free filtrates.

Work has been done to modify the method or to devise similar ones based on the silver iodate reaction (*6–13*). These modifications include the simultaneous precipitation of protein and reaction with $AgIO_3$, as well as changes in the titrimetric and colorimetric (or spectrophotometric) procedures for determination of the liberated iodate. The present study is an extension of Sendroy's method to the use of spectrophotometry for the analysis of blood, plasma, serum, or packed red cells, on a micro scale. An effort has been made to utilize glassware and equipment normally present in a clinical chemistry laboratory. Some of the suggestions for improvement of the iodate procedure have been incorporated into the present easy, sensitive, and reproducible method.

## Reagents

1. *Standard 0.120 M chloride solution* Analytical grade KCl (8.946 gm./L.), dried at 110-120° is used. Working standards of 40 and 60 mM for the analysis of red cells, and of 75, 100, and 120 mM for the analysis of blood and serum are required. The first four solutions are made by the accurate addition of 10, 5, 3, and 1 ml. of water, respectively, to 5 ml. of the stock solution.

2. *Phosphoric acid-sodium tungstate solution* This contains 0.30 M $H_3PO_4$ and 2.0% sodium tungstate. Twenty grams of $Na_2WO_4 \cdot 2H_2O$ (Baker's reagent sodium tungstate "according to Dr. Folin") are dissolved in about 800 ml. of water. Most samples of sodium tungstate require neutralization of an appreciable carbonate contamination. Syrupy phosphoric acid (reagent grade) is added dropwise until a drop of the tungstate solution is acid to an external indicator, such as phenol red solution (pH, 6–7) or an appropriate indicator paper. Then 20 ml. more of phosphoric acid is added, and dilution made to 1 L. This single stable protein precipitating solution is used for all analyses and for obtaining the standard chloride curve.

3. *Silver iodate* Merck's reagent "suitable for the determination of chloride" (*14*) is used.

4. *Reagent grade potassium iodide*

5. *0.020 N thiosulfate solution* Five grams of $Na_2S_2O_3 \cdot 5H_2O$ and 1.0 gm. of anhydrous $Na_2CO_3$ are dissolved in 1 L. of distilled water. After at least 24 hr., the thiosulfate concentration is tested and adjusted as follows. An accurately measured 5.0-ml. sample of 0.030 N $KIO_3$ solution (1.07 gm. of $KIO_3$ per L.) is mixed with 1 ml. of the phosphoric acid-sodium tungstate solution. One milliliter of 5% KI solution is added and the thiosulfate immediately run in from a 10-ml. buret until the solution is colorless. The volume of thiosulfate required should be 7.50 ml. If it differs from this by more than 0.20 ml., adjustment is made by the addition of water or thiosulfate to the stock solution until the titre is between 7.30 and 7.70 ml. The solution is sufficiently stable to be used for several months.

6. *KI-thiosulfate diluent for color development* This contains 0.5% KI and $4.0 \times 10^{-4}$ N $Na_2S_2O_3$. It is prepared with 5 gm. of KI and 20.0 ml. of the stock 0.020 N $Na_2S_2O_3$ in 1 L. of solution.*

## Procedure

The following procedure, described for the analysis of plasma or serum, is applied without change to whole blood analysis. Red cells are also analyzed by the same procedure when standard chloride and KI-$Na_2S_2O_3$ solutions of appropriate concentrations are used.

### Preparation of Protein-free Filtrate

A 5-ml. centrifuge tube or a 13 × 100-mm. test tube is prepared for each standard, and in duplicate for each unknown. In each tube 0.50 ml. of water is placed. It is essential that this volume measurement be the same in all tubes, for which reason a single Lang-Levy constriction pipet (commercially available) should be used. Into each tube, by use of an accurate Sahli pipet, 0.020-ml. samples of standard chloride solutions (75, 100, and 120 mM) or plasma or serum are transferred. The volume is adjusted to the mark by repeated touching of the tip of the pipet to the surface of a sheet of bond paper.† The pipet is rinsed by

*These dilute thiosulfate solutions gradually decrease in normality. Although they may ordinarily be used for a period of nearly a week, it is preferable that they be freshly prepared. The same thiosulfate solution must be used in the analysis of each standard and unknown.

†The common practice of using the surface of one's finger or hand for this purpose should be avoided.

repeated suction of the water into it and expulsion back into the tube. Only minimal quantities of solution are allowed to adhere to the pipet as it is removed from the tube, the contents of which are then mixed by gentle shaking. With the same precautions indicated for the addition of water, 0.50 ml. of the phosphoric acid-tungstate solution is added. The tubes are covered tightly (a small piece of Parafilm is very convenient) and shaken vigorously for about 1 min. The protein-containing tubes are centrifuged 3–5 min. at about 800g (2200 rpm in an International No. 2 centrifuge). The clear supernatant fluid is decanted into a 5-ml. centrifuge tube.

### Reaction with Silver Iodate

To each tube containing chloride solution or protein-free filtrate, 6–10 mg. of $AgIO_3$ is added. The tubes are sealed with Parafilm, shaken vigorously 40–60 sec., and centrifuged 3–5 min. at approximately 800g. Contamination by solid silver salts in the subsequent transfer of supernatant solution from the centrifuge tubes is avoided as follows. When the desired speed is reached, centrifugation is stopped and the sealed tubes are gently tilted to wash off any solids adhering to the rims and walls of the tubes. The centrifugation is then resumed. The iodate in these solutions, whether they be immediately separated from the solid silver salts or left standing over them, is stable and has been analyzed without observable change over a period of 24 hr.

### Color Development

A Folin-Ostwald pipet is used to transfer 0.5 ml. of the clear supernatant solution from the silver iodate reaction into 10.0 ml. of the KI-$Na_2S_2O_3$ diluent solution in a test tube.* The pipet is rinsed† with the solution by repeated suction of the diluent from, and expulsion back into, the tube. Finally, the tube is capped with Parafilm, inverted, and the contents mixed. The absorption is read at 400 m$\mu$ in 1-cm. cells in a Beckman (Model DU) or other suitable spectrophotometer. It is convenient to set the spectrophotometer at zero absorbance with the standard chloride solution of lowest concentration in the light path.

*Should the analyst find it necessary or desirable to use the cotton plug filter originally recommended (*3*) for the transfer of the centrifuged solution, chloride contamination from moist fingers during the insertion of the plug should be avoided.

†Although Folin-Ostwald pipettes are calibrated to deliver by blowout, their use for washout, uniformly applied to the delivery of both standards and unknown, leaves unimpaired the accuracy of the analysis described. On the other hand, quantitative transfer of the sample to the diluent solution by blowout, is extremely difficult.

By this means, only the *excess* of concentration above that of this standard is measured in all of the other solutions.

### Calculations

Since unknown and standard solutions all have identical treatment at every stage of the analysis, the chloride concentration of the unknown is read directly from the curve of absorption vs. concentration of the standards. *It is important to use standard solutions of concentrations both lower and higher than those of the unknown solutions.*

The following is an example of the calculation for the analysis of a sample of human serum. When the absorbance of the 75 mM standard was set at zero, the absorbancies obtained for the 100-mM and 120-mM KCl solutions, and the serum, were 0.345, 0.621, and 0.410, respectively. From the absorbance change of $0.276/20 = 0.0138$ U./mM $Cl^-$ for the standards, the concentration of chloride in the serum sample was calculated to be: $75 + (0.410/0.0138) = 104.7$ mM.

### Analysis of Whole Blood and Red Cells

Whole blood may be analyzed by the procedure described without change. The analysis of red cells requires standard KCl solutions of 40 and 60 mM and a color development solution containing 0.5% KI and $2.0 \times 10^{-4}$ N $Na_2S_2O_3$. No other change is needed.

## Experimental Results

### The Species of Iodine in Solution

In the silver iodate method carried out by titrimetric and colorimetric or spectrophotometric technics, the iodate liberated is measured as the iodine formed on reaction with iodide ion in acid solution. In the original colorimetric method (*5*), the blue color of the starch-iodine complex was used. This procedure was later converted to a photoelectric technic in which the yellow-brown color of iodine in potassium iodide solution was measured. Subsequently (*9*), a wave length of 420 m$\mu$ was used for convenience, although maximum absorption, attributed to the formation of the addition compound, $KI_3$, was found to be at 355 m$\mu$ (*8, 9*). In fact, Jones and Kaplan (*15*) had shown that there was a reversible equilibrium between molecular iodine and iodide ion with the formation of the univalent triiodide ion. They found the dissociation constant of the triiodide ion, $K = (I_2)(I^-)/(I^-_3)$, to be $1.40 \times 10^{-3}$ at 25°.

### Absorption Curves of Iodine and Triiodide Ion

In order to determine the optical properties of the forms of iodine (molecular $I_2$ and $I^-_3$ ion), the absorption spectra of iodine in various solutions similar to those used by Awtrey and Connick (*16*) have been

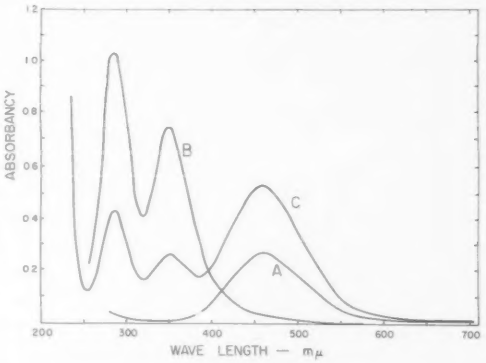


**Fig. 1.** Absorption spectra of iodine solutions. Curve A: $3.5 \times 10^{-4}$ M iodine in 0.13 M $H_3PO_4$ + 0.005 M $KIO_3$. Curve B: $3.2 \times 10^{-5}$ M iodine in 0.13 M $H_3PO_4$ + 0.01 M KI. Curve C: $8.1 \times 10^{-4}$ M iodine in distilled water. Each curve was obtained by use of a Beckman DU spectrophotometer, 10-mm. light path, and a blank solution containing all components except iodine.

determined in the visible and near ultraviolet. The absorption spectra in Fig. 1 show that a solution containing only molecular iodine, $I_2$, and essentially no iodide ion (Curve A) has an absorption maximum at 460 m$\mu$, but no absorption near 350 m$\mu$. On the other hand, when essentially all of the iodine is in the form of triiodide, $I^-_3$ (Curve B), there are intense absorption maxima at 287 and 353 m$\mu$, but no maximum in the region of 460 m$\mu$. Pure resublimed iodine dissolved in redistilled water (Curve C) produces absorption maxima at 460, 353, and 287 m$\mu$, indicating, as a result of hydrolysis, the presence of both $I_2$ and $I^-_3$. The molar absorption index, $a_M$, of the triiodide ion at 350 m$\mu$ was observed to be 25,500, whereas the value for $I_2$ at 460 m$\mu$ was found to be 748.* Indeed, the molar absorption index of $I^-_3$ is always greater than that of $I_2$ even at the absorption maximum of the latter (*16*).

Thus, it is apparent that over the span of wave length 350–460 m$\mu$, the species of iodine which contributes most to the absorption is the triiodide ion. It is, therefore, important to determine the concentration of iodide required to give maximal absorption with a given amount of iodine. Figure 2 shows that maximal absorption at 350 m$\mu$ is reached with $5 \times 10^{-2}$ M iodide, about 2000 times the molar concentration of iodine. The lower concentration of $3.0 \times 10^{-2}$ M (0.5% KI)

*These values are in excellent agreement with those reported by Awtrey and Connick (*16*) and those recalculated from the data of Custer and Natelson (*17*).

has been chosen for the present method. Under the conditions of analysis, this concentration of iodide keeps about 95% of the iodine in the strongly absorbing, nonvolatile triiodide form. The spontaneous formation of iodine by air oxidation of acidified iodide solution, is also minimized (Curve B, Fig. 2).

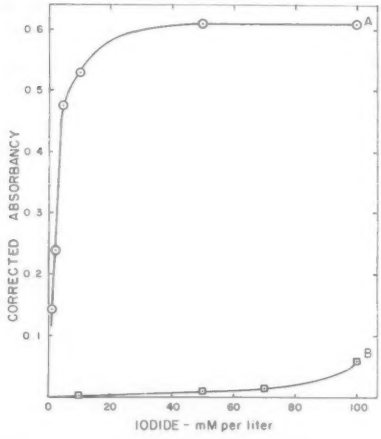


**Fig. 2.** Effect of iodide concentration (Curve A) on the absorption at 350 mμ, of $2.38 \times 10^{-5}$ M iodine solutions in 0.2 M $H_3PO_4$. Curve B is for blank solutions from which the iodine was omitted. Length of light path, 10 mm.

### Sensitivity of Absorption Measurements

The high molar absorption index of $I^-_3$ at 350 mμ would make it necessary to dilute a 100-mM chloride solution such as serum approximately 15,000 times in order to measure all of the chloride and to obtain absorbance below 0.7 at this wave length. Furthermore, the sensitivity, *i.e.*, $\Delta$ A/$\Delta$ $Cl^-$, is only approximately 0.004 absorbance unit for a change in chloride concentration of 1 mM. At lower dilution, approximately the same sensitivity may be obtained from the absorption at 400 mμ. The sensitivity is increased 10-fold or more when a constant amount of the iodine is made to react with thiosulfate in the manner suggested by Stiff (*11*). In the present procedure, with dilution of about 1000 and measurement at 400 mμ, the sensitivity is approximately 0.016 absorbance unit per millimole change in chloride. Typical standard curves, measured with two instruments, are shown in Fig. 3.*

*A linear relationship was observed between absorption and chloride concentration when measurements were made with the Beckman spectrophotometer within the concentration range shown. When the Coleman Jr. instrument was used, the standard curve was not linear, probably owing to the greater spread of wave length of incident light of this instrument.

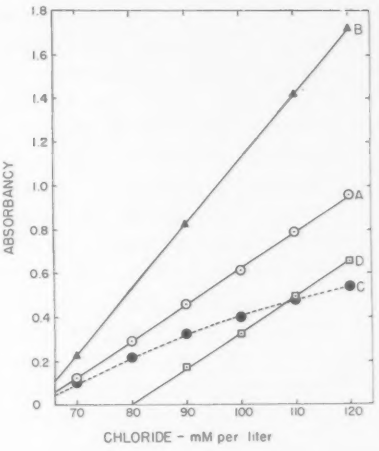


**Fig. 3.** Change of absorption with chloride concentration of standard solutions. For the Beckman spectrophotometer, 10-mm. cell: Curve A, reagent blank (KI-$Na_2S_2O_3$) set at zero, 400 mμ; Curve B, reagent blank (KI-$Na_2S_2O_3$) set at zero, 385 mμ; and Curve D, 80 mM Cl standard set at zero, 400 mμ. For the Coleman Jr. spectrophotometer, 12 × 75 mm. cuvet: Curve C, reagent blank (KI-$Na_2S_2O_3$) set at zero, 400 mμ.

## Results of Analyses of Serum and Whole Blood by Present Method

Several samples of pooled human serum were analyzed as prescribed in the foregoing and by the Van Slyke nitric acid digestion method (*18*). Table 1 shows that mean values for the present method average about 1% higher than results obtained by digestion. This mean increase is more than 3 times the standard error of the mean as calculated for small samples (*19*) and is, therefore, considered statistically significant ($P = 0.01$). This difference confirms earlier work in which

**Table 1.** Comparison of Chloride Analyses of Human Serum by Two Methods

| | *Method* | | |
|---|---|---|---|
| *Sample* | *Digestion (mM/L.)* | *Spectrophotometric (mM/L.)* | *Ratio** |
| 1 | 102.6 | 103.9 | 1.013 |
| 2 | 104.9 | 105.3 | 1.004 |
| 3 | 105.0 | 105.1 | 1.001 |
| 4 | 104.2 | 104.8 | 1.006 |
| 5 | 102.9 | 104.6 | 1.017 |
| 6 | 105.2 | 107.7 | 1.024 |
| 7 | 104.8 | 106.0 | 1.011 |
| 8 | 104.3 | 104.0 | 0.997 |
| 9 | 104.3 | 105.4 | 1.011 |
| | | | Av. 1.009 |

*Spectrophotometric divided by digestion values.

the iodate of tungstic acid filtrates was measured by gasometric and titrimetric means (*3, 4, 10*). As suggested previously (*3*), it seems unlikely that the observed difference lies in the extent of serum dilution; essentially the same value has been found by all three methods over a range of dilution from 10- to 50-fold. Table 2 shows similar comparative values for analyses of human whole blood. A difference of 1% is likewise apparent but this is not statistically significant. Furthermore, none was previously observed for tungstic acid filtrates (*3*).

The precision of the present procedure was tested with 14 separate analyses of a sample of human serum (Sample 9, Table 1). A mean chloride value of 105.4 with a standard deviation of ±0.85 mM was found. Replicate analyses of whole blood and of red cells have shown that the precision of the method is nearly the same for these materials, also. The major factor determining the variation appears to be the accuracy with which the small 0.02-ml. samples can be measured.

**Table 2.** Comparison of Chloride Analyses of Human Blood by Two Methods

| | *Method* | | |
|---|---|---|---|
| *Sample* | *Digestion (mM/L.)* | *Spectrophotometric (mM/L.)* | *Ratio** |
| 1 | 79.1 | 82.3 | 1.040 |
| 2 | 83.2 | 84.3 | 1.013 |
| 3 | 71.4 | 70.7 | 0.990 |
| 4 | 81.4 | 82.5 | 1.014 |
| 5 | 83.0 | 84.8 | 1.022 |
| 6 | 87.9 | 87.4 | 0.994 |
| | | | Av. 1.012 |

*Spectrophotometric divided by digestion values.

### Effect of Proteins on the Silver Iodate-Chloride Reaction

The silver iodate method was primarily developed by Sendroy (*3*) as a means of direct determination of chloride in plasma, serum, and urine, *without recourse to deproteinization,* although technics were also outlined for the analysis of protein-free filtrates of plasma and whole blood.

Van Slyke and Hiller's modification (*10*), which was subsequently used by other workers (*11–13*), was to carry out in plasma samples, the simultaneous precipitation of protein and the silver iodate reaction. It seemed that such a procedure might be advantageous in its extension to the analysis of blood and red cells, particularly by the spectro-

photometric technic presently developed. To test this possibility a fresh sample of heparinized dog blood was used. The whole blood, and the plasma and red cells obtained therefrom, were each analyzed in triplicate (1-ml. samples) by the Van Slyke (*18*) digestion method as modified by Eisenman (*20*). Each sample was also analyzed by triplicate spectrophotometric measurement of the phosphoric-tungstic acid supernatant fluid obtained in two ways: in Method A, the precipitated protein was separated from the fluid prior to the addition of $AgIO_3$; in Method B, the $AgIO_3$ was added in the presence of the precipitated proteins.

The results, in Table 3, show that chloride values for blood and its two components, obtained by treating the protein-free filtrate with $AgIO_3$ (Method A), correspond closely to those found by the digestion method. By contrast, values obtained when the protein precipitate is present during the reaction with $AgIO_3$ (Method B) are increased roughly in proportion to the protein (cell) concentration. In comparison with the digestion method, the chloride values obtained for plasma, whole blood, and cells of this particular dog blood sample, treated with $AgIO_3$ in the presence of the precipitated protein, were respectively 4, 18, and 47% too high. Similar comparative analyses were carried out to ascertain the effect of the precipitated protein in samples from other species. Measurements were made by titration or spectro-

**Table 3.** COMPARATIVE CHLORIDE ANALYSES OF DOG BLOOD BY TWO METHODS

| | *Method* | | |
|---|---|---|---|
| | | *Spectrophotometric** | |
| *Sample* | *Digestion (mM/L.)* | *Method A (mM/L.)* | *Method B (mM/L.)* |
| Blood No. 1 (Het. = 0.435) | | | |
| Whole blood | 87.8 | 89.0 | 103.3 |
| Plasma | 114.7 | 115.8 | 119.2 |
| Red cells | 52.5 | 52.2 | 77.3 |
| Red cells (calculated)† | 52.9 | | |
| Blood No. 2 (Het. = 0.373) | | | |
| Whole blood | 82.1 | 82.1 | |
| Serum | 104.8 | 106.0 | |
| Red cells | 44.0 | 44.7 | |
| Red cells (calculated)† | 44.0 | | |

*Method A: according to the procedure described for the present method. $AgIO_3$ was added *after* the precipitated proteins were removed. Method B: $AgIO_3$ was added in the presence of the precipitating proteins.

†Calculated from values for hematocrit, and digestion analyses of whole blood and plasma.

**Table 4.** Comparison of Chloride Analyses Showing Results of $AgIO_3$ Reaction in the Presence of Precipitated Protein

| *Sample* | *Method of iodate analysis* | *Digestion (mM/L.)* | *$AgIO_3$ reaction** | |
|---|---|---|---|---|
| | | | *Method A (mM/L.)* | *Method B (mM/L.)* |
| Human blood | Titrimetric | 83.0 | 84.7 | 89.1 |
| Human plasma | Titrimetric | | 100.0 | 102.7 |
| Ox serum | Titrimetric | | 101.8 | 104.8 |
| Ox blood | Titrimetric | | 84.0 | 98.3 |
| Ox red cells | Titrimetric | | 58.3 | 76.7 |
| Ox serum | Spectrophotometric | 100.8 | 100.1 | 102.3 |
| Ox serum | Spectrophotometric | | 102.9 | 106.4 |
| Ox serum | Spectrophotometric | | 99.1 | 101.3 |
| Ox blood | Spectrophotometric | | 84.3 | 95.9 |
| Ox blood | Spectrophotometric | | 82.4 | 90.3 |

*Method A: According to the procedure described for the present method. $AgIO_3$ was added *after* the precipitated proteins were removed. Method B: $AgIO_3$ was added in the presence of the precipitating proteins.

photometry. Table 4 likewise shows that in all cases values obtained in the presence of the precipitated protein were higher than those from the corresponding protein-free filtrates, which in turn, were in substantial agreement with digestion values. The results for plasma and serum in Tables 3 and 4 indicate the effect of the presence of the precipitated proteins in the mean increase in values of 2.83 mM/L. by Method B as compared with Method A. This is more than 12 times the standard error of the mean for this group of analyses, and must be regarded as being most highly significant (*19*). The differences for whole blood and cells are obviously valid. The effect noted apparently varies with the species, being least for human blood, and increasing with the red cell concentration. On the basis of the evidence given, the present method requires that the protein precipitate be removed before the addition of $AgIO_3$. This step is mandatory for the analysis of whole blood or red cells.

## Discussion

Previous applications of the silver iodate method, with the exception of that to urine analysis (*3, 21*), have all been carried out in solutions of at least 3 mM chloride, to eliminate a correction for $AgIO_3$ solubility (*3*). Such a restriction is not important for the present procedure because the amount of $AgIO_3$ in solution is controlled by the analysis of

standard chloride solutions of approximately the same concentration as the unknown. When all standard and unknown solutions are treated alike throughout the procedure, factors such as the extent of dilution, temperature, time of mixing or standing, chloride contamination in reagents, and the solubility of $AgIO_3$, do not affect the analysis.

In the authors' experience, the suggested simplification of the iodate method (*10*) by simultaneous precipitation of plasma protein and reaction with $AgIO_3$ always yields higher values than those obtained by nitric acid digestion of the sample. Van Slyke and Hiller's data (*10*) indicate such results to be 1.7% too high for dog plasma whereas our results for the same (and the plasma of other species) are about 4% high. The extension of their procedure to the analysis of samples containing a high concentration of protein, such as blood, red cells, or other tissues, is evidently not feasible (Tables 3 and 4). Although the exact reason for this effect of precipitated protein has not been determined, it is at least partially a result of the binding of silver ions by the proteins, and, to a much lesser extent, of a reduction of iodate to iodide. Recent experimental evidence to this effect has been obtained as follows. The proteins were precipitated from samples of human blood according to the present method, then washed free of chlorides with successive portions of the precipitating solution. Upon the addition of $AgIO_3$, iodate was liberated in amount approximating the excess found for chloride in human blood when the steps of deproteinization and $AgIO_3$ treatment were combined (Table 4). On the other hand, as shown above, chloride values found for phosphoric-tungstic acid filtrates of plasma, whole blood, or red cells, from which the protein was separated and removed prior to treatment with $AgIO_3$, are in substantial agreement with the values obtained by digestion.

Annino (*22*) has found that the native proteins increase the chloride values for serum when measured by the mercurimetric method of Schales and Schales (*23*). Such results indicate that mercuric ion reacts not only with chloride, but to some degree with serum proteins. Where there is evidence of significant error in chloride methods involving the use of mercuric or silver ions in the presence of protein, the latter should be not only precipitated, but removed prior to the continuation of the analysis.

King and Bain (*13*) were unable to measure chloride in standard NaCl solutions with silver iodate in the presence of sodium tungstate. No such difficulty has been encountered in the present work. The acid concentration used is higher than necessary to remove protein, but was

so chosen in order to provide sufficient acid for the subsequent color development.

The iodate may be measured by the gasometric procedure for which no standard or solution of known composition is required. When iodate is measured by reaction with excess KI in acid solution, the iodine released may be titrated with thiosulfate or measured spectrophotometrically. A combination of the latter two analytical steps has been used, as advocated by Stiff (*11*), who pointed out the advantages of the increased sensitivity obtained when most of the iodine was first reduced with thiosulfate, with resultant lower dilution of the sample. In his method, a dilute thiosulfate solution of exactly known concentration was used for partial reaction with the iodine. Such solutions are rather unstable and require constant preparation or restandardization. In the present method, a constant volume of a similar solution, whose thiosulfate concentration needs to be set only within certain limits, is used for all standard and unknown solutions. In effect, the dilution with a known amount of thiosulfate has so been chosen that the physical dilution is about 1000, but the "chemical dilution" caused by reaction of iodine with thiosulfate accounts for about 70% of the total chloride. Furthermore, potassium iodide has been added to the thiosulfate solution in order to simplify the color development. The amount of excess iodine observed by this procedure is the same as that found when all of the iodine is liberated before the addition of thiosulfate.*

The method as described is an attempt to provide adequate sensitivity over the range of concentration of chloride encountered in the analysis of blood, plasma, or cells. For special purposes, it is possible to alter the sensitivity of the measurements greatly by changing the total dilution of the sample, by altering the amount of iodine reacting with thiosulfate, and by reading the absorption at other wave lengths. The conditions prescribed are a compromise, since increasing the sensitivity further makes the technic considerably more demanding.†

Measurement of the absorption at 400 m$\mu$ has been chosen for routine analysis. The sensitivity of the method is doubled by reading absorbance at 385 m$\mu$, and is about 5 times as great if measurements are made at 350 m$\mu$. However, readings at this wave length will seldom be

*Thiosulfate does react with iodate in the absence of iodide ion, but this reaction is very slow in comparison with the rate of reaction of iodide with iodate in acid solution.

†Although the molar absorbancy index of $I^-_3$ is higher at 287 m$\mu$, this wave length can not be conveniently used because quartz optics are required and because iodide and tungstate ions, present in excess in these solutions, seriously interfere with absorbancy at this wave length.

practical since a much more critical adjustment of the thiosulfate concentration is then required.

We have found the molar absorption index of $I^-_3$ at 350 m$\mu$ to be approximately 2/3 that of the blue starch-iodine complex at 600 m$\mu$. The starch-iodine complex has not been used in this work, however, because increased sensitivity was not needed and because reproducible color development of the starch-iodine complex is much more difficult (*7*). Although the method is described for 0.02-ml. samples, it is obvious that larger samples may be used if desired. It is also possible to change the dilution, both in the original protein-free filtrate preparation and in the color development. When such modifications are made, care must be taken to use $AgIO_3$ sufficiently in excess, and to use a color development solution which leaves a small amount of excess iodine in the most dilute standard and unknown solution.

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# The Accurate Gasometric Determination of the Oxygen-Hemoglobin Equilibrium in Dilute Solutions of Sheep Hemoglobin

*F. J. W. Roughton*

**Apparatus and technique are described for the accurate gasometric macrodetermination of the oxygen-hemoglobin equilibrium in dilute solutions containing around 0.15 gm. Hb/100 ml., equivalent to blood diluted 100:1.**

**At the bottom of the equilibrium curve, i.e., in the range 0–2% $HbO_2$, the method is accurate to ± 0.05% $HbO_2$. Over the main part of the curve the mean accuracy is ± 0.5% $HbO_2$. From such data, together with indirect kinetic information as to the character of the extreme top of the equilibrium curve, it has been possible, in the case of dilute sheep Hb solutions, to compute all four constants in Adair's general intermediate compound equation for the equilibrium between oxygen and hemoglobin.**

**Comparison of the results at pH 7.1 and 9.1 suggests that the first two oxygen molecules combine independently at both pH's. Interaction sets in at the third and more markedly at the fourth stage, but there are detailed differences in the degrees of interaction at these last two stages at the two pH's.**

**It is hoped that these equilibrium data in dilute sheep Hb solution will be of service in interpreting kinetic experiments on the rates of the reactions of oxygen and hemoglobin, which so far can only be carried out in dilute solutions of the latter.**

THE OXYGEN REACTION of hemoglobin, as Wyman and Allen (*1*) have well observed, is "a matter of great significance in its own right, representing as it does one of the fundamental phenomena of biochemistry, and any light which can be thrown on it will lead to a deeper understanding of a major physiological process." It is not then surprising that, throughout the present century, an immense amount of quantitative work has been carried out upon the equilibrium of this reaction: in the last 30 years or so the kinetics of the reaction have also been much studied, though by far fewer workers. A recent review was made by Gibson (*2*).

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The classical method of studying the equilibrium between oxygen and hemoglobin has been to equilibrate either whole blood or a solution of hemoglobin with gas mixtures containing a series of appropriate partial pressures of oxygen and carbon dioxide, and then to measure (usually by gasometric or spectrophotometric methods) the percentage saturation of the hemoglobin with oxygen when equilibrium has been attained. Tonometers of the Barcroft type have customarily been used for the equilibration, and the gasometric estimation of the oxygen content of the equilibrated solution has, for the past 30 years or more, generally been made by means of the Van Slyke-Neill constant volume manometric apparatus. Recently Van Slyke and Plazin (*3*) have described a most attractive micro-modification of the latter. With such methods the percentage saturation has been measurable to about ± 1% over the range of main interest to the physiologist, i.e., 10–98% $HbO_2$. This degree of accuracy is ample for most clinical and physiological purposes.

However, from the physico-chemical viewpoint, Roughton has stressed that, for a proper quantitative test of Adair's famous theory (*4*) that the reaction of oxygen with hemoglobin takes place in four intermediate stages, it is necessary also to have results of some 20 times greater accuracy at the extreme bottom (i.e., 0–2% $HbO_2$) and the extreme top (i.e., 98–100% $HbO_2$) of the oxygen-hemoglobin equilibrium curve. Roughton and his colleagues (*5, 6*) have met these needs by devising special methods which they have applied successfully to fairly strong solutions of sheep hemoglobin (4 gm. Hb/100 ml. and higher).

The next step in the physico-chemical program has been to extend these technics to much more dilute hemoglobin solutions (0.4 to 0.02 gm./100 ml.), so that the accurate equilibrium data on the $O_2$-Hb reaction can be correlated with the measurements on the rates of the rapid reactions of oxygen with hemoglobin, which—with the presently available kinetic technics—can only be made with very dilute solutions of hemoglobin or suspensions of red cells. The present paper describes an adaptation of the gasometric technic to the study of the $O_2$-Hb equilibrium in dilute solution, the accuracy now attainable being ± 0.05% $HbO_2$ over the range 0–2% Hb, and ± 0.5% $HbO_2$ over the range 10–98% $HbO_2$. I have not yet obtained a gasometric accuracy of ± 0.05% $HbO_2$ at the top of the curve for dilute Hb solutions, but this gap—from the physico-chemical viewpoint—has, it is believed, been filled by an indirect method described later in this paper. In devising these gasometric procedures—as, of course, in all my gasometric work dur-

ing the past 35 years—I owe a huge debt to the work and encouragement of Dr. D. D. Van Slyke and his school: I hope that some of the special technical details, which it has been necessary to introduce, may interest my old master, and thus form a slight though quite inadequate personal tribute to him from me on the happy occasion of his attaining 80 years of age.

## Experimental Procedures

### Accurate Gasometric Determination of $O_2$ Equilibrium Curve in 0–2% $HbO_2$ Range

#### Source of Hemoglobin

The most common species of mammalian hemoglobin used in the quantitative study of the oxygen equilibrium have been human, horse, and sheep. In the present work sheep Hb has been preferred for the following reasons:

1. It shows no tendency to split into half-molecules at the dilutions (0.1–0.2 gm./100 ml.) and over the pH range (7.0–9.1) studied.

2. It is more stable against methemoglobin formation and other kinds of inactivation in vitro than are many other species of mammalian blood.

3. It naturally contains only very small amounts of carbon monoxide hemoglobin ($< 0.1$ volumes %). In the final equilibrated solutions HbCO is not usually detectable, which is important in view of the known effect of HbCO (and also of methemoglobin) on the oxygen equilibrium curve of hemoglobin.

4. It can be obtained naturally in one of two genetically distinct Types (A or B), selected sheep belonging to Type A, to Type B, or having mixed hemoglobins of both types.*

Usually about 200 ml. of heparinized blood has been drawn from a selected sheep, the red cells washed three times with saline solution (0.9 gm. NaCl/100 ml.) and then laked by adding two parts of distilled water. After 15 min. standing, the red-cell ghosts are removed by 15 min. centrifugation (25000 g). The supernatant Hb solution is then deoxygenated by repeated shaking with nitrogen and by vacuum in a 2.5-L. bottle; it is then mixed with one third its volume of deaerated buffer (borate or phosphate). The oxygen-free Hb solution (residual $HbO_2 < 1.5\%$), is transferred to a 1-L. bottle, closed with a rubber

*In most of the present work, Type A hemoglobin has been used, from sheep kept at the Institute of Animal Physiology at Babraham, near Cambridge, England, to the authorities of which I am very grateful.

stopper through which pass two glass leads (one to the top and the other to the bottom of the bottle, both being closable by attached rubber tubes with screw clamps), and stored in the inverted position under a positive pressure of nitrogen at 1–4°. This stock solution is diluted with buffer solutions as required (and as described later), and can be used for up to one week without significant change in the properties of the hemoglobin. The hemoglobin was not further purified, in view of the attendant dangers of methemoglobin and/or other types of inactivation. As Barcroft (*7*) wrote, "It is one of the ironies of hemoglobin that the greater the pains at which you are to make it pure the greater is the extent to which you are likely to contaminate it by the formation of the inactive variety."

### Manometric Determination of % $HbO_2$ to ± 0.05% $HbO_2$ in 0–2% $HbO_2$ Range

The technic is basic to all the work presented here, and warrants full description. An ordinary Van Slyke manometric chamber, with the usual graduation marks at 0.5, 2.0, and 50 ml., is cut about 1 cm. below the 2-ml. mark and joined below to a 300-ml. Barcroft tonometer, to the tap of which is attached a bulb of about 15-ml. capacity, as in the Harington-Van Slyke (*8*) manometric chamber (Fig. 1A). Since the top

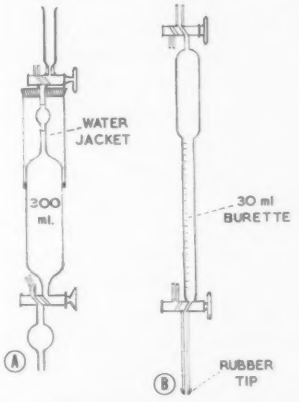


**Fig. 1A.** Van Croft manometric chamber. **B.** Tonometer, with attached buret for deaerated ferricyanide reagent.

of this apparatus is attributed to Van Slyke and the bottom to Barcroft, we have in laboratory slang christened it a "Van Croft"—a term Dr. Van Slyke has blessed and with which I feel the late Sir Joseph Barcroft would also have agreed. About 150 ml. of the dilute Hb solution, whose $O_2$ content is required, is evacuated with acid-ferri-

cyanide solution; after absorption of evolved $CO_2$, the residual gas is measured at the 0.5-ml. mark, $p_{1.5}$. The percentage oxygen is then determined by ejecting the whole, or a fraction, of the extracted gas into a Scholander-Roughton (Fig. 3A) syringe-capillary (*9*) and analyzing the bubble for $O_2$ in the usual way. The vacuum reading, $p_{2.5}$, (with only water vapor in the chamber) is taken at the 0.5-ml. mark, and the $O_2$ content then calculated from the pressure difference ($p_{1.5}$–$p_{2.5}$) in the same way as was done by Roughton and Root (*10*) in their analogous estimations of very small amounts of carbon monoxide in blood, the present technic—after the measurement of $p_{1.5}$—being exactly similar to theirs.

The analysis of the $O_2$ content of the extracted gas by means of the syringe capillary instead of the more usual procedure of running oxygen absorbent into the Van Croft chamber has the advantages of (1) avoiding "c" corrections, and (2) eliminating errors from differences of surface tension and/or vapor pressure (which differences may arise when the composition of the fluid in the chamber is not the same at the pressure reading $p_{1.5}$ as at the reading $p_{2.5}$). With small amounts of extracted gas it is indeed possible, in the present procedure, to measure the value of $p_{1.5}$–$p_{2.5}$ to within ±(0.1–0.2) mm. Hg, corresponding to the limit of visual accuracy of reading of the mercury meniscus at the 0.5-ml. mark. It is necessary, however, in order to achieve the necessary accuracy in the $O_2$ analysis by the syringe-capillary, to keep the dissolved $N_2$ content of the Hb solution as low as possible. For this reason the Hb solutions are equilibrated with low pressures of oxygen *in vacuo* rather than in nitrogen.

The mercury reservoir attached to the Van Croft arrangement requires about 350 ml. of mercury, i.e., about 4 times as much as usual. To save the strain of constantly raising and lowering such a large weight it is convenient to have the top of the reservoir closed by a 3-way tap, one exit of which can be connected to the air and the other to a vacuum.

Controls have shown that with small amounts of evolved oxygen (0–20 $\mu$l. S.T.P.) the method is usually accurate to ± 0.10 $\mu$l. and is thus on a par with the micromethod of Van Slyke and Plazin (*3*) and with Roughton and Root's carbon monoxide determinations (*10*). With 150 ml. of dilute hemoglobin solution of 0.15 gm. Hb/100 ml. (equivalent to whole blood diluted 100:1—the standard dilution used in these studies), an absolute error of ± 0.1 $\mu$l. corresponds to an error of ± 0.033% $HbO_2$.

Had the Van Slyke-Plazin microtechnique been available at the time when the Van Croft procedure was first embarked upon (1957), I have no doubt that use would have been made of some of its features.

### Reagents Required

*1% $KHCO_3$ solution*

*1% lactic acid solution* Pure lactic acid is diluted 100:1 with water.

*32% $K_3Fe(CN)_6$ solution* 32 gm. of $K_3Fe(CN)_6$ is ground in a mortar with 86 ml. water, the resulting volume being 100 ml.

*1 M $KH_2PO_4$ solution*

*Acetate buffer* 70 gm. of sodium acetate ($NaC_2H_3O_2 \cdot 3H_2O$) is dissolved in 100 gm. of water, and 15 ml. of glacial acetic acid added.

*10% NaOH*

*40% NaOH*

*$Na_2S_2O_4$—anthraquinone β-sulfonate solution for oxygen absorption* 16 gm. $Na_2S_2O_4$, 14 gm. KOH and 3 gm. anthraquinone β-sulfonate are made up to 100 ml. with water in a stoppered glass vessel of capacity just greater than 100 ml.

### Van Croft Procedure

About 5 ml. of water with 3–4 drops of octyl alcohol are shaken for 3 min. in the evacuated main chamber and are then drawn down into the vacuous 15-ml. cup, where they are shaken for a further 3-min. period. The extracted gas is quantitatively ejected through the top Van Croft tap and the water evacuated and shaken again in the main chamber, but not in the lower cup. No bubble of air should be seen on compression to atmospheric pressure after the second extraction; this provides a good check that the Van Croft chamber is properly free from gas leaks.

One milliliter of 1% $KHCO_3$ and 2 ml. of 1% lactic acid are then added to the 5 ml. of air-free water, and the resulting 8 ml. of solution shaken for two 3-min. periods in the vacuous chamber, the evolved gas being quantitatively ejected each time. About 3 ml. of gas (S.T.P.) is extracted by the first shaking, consisting of about 99% $CO_2$ and 1% air (from the initial dissolved air contents of the $KHCO_3$ and lactic acid solutions), whereas the smaller amount of gas extracted by the second shaking is at least 99.99% pure $CO_2$. The quantitative ejection of this highly pure $CO_2$ through the top tap of the Van Croft washes out any minute oxygen-containing bubbles which might still be adhering to the bore of the tap and its capillary leads, and which would otherwise

cause error at a later stage. The 8 ml. of completely deaerated solution in the Van Croft chamber is later used to flush out the connections between the chamber and the tonometer in which the dilute Hb solution has been equilibrated, and a sample of 150–160 ml. of the latter drawn into the Van Croft with the full anaerobic precautions to be described below.

Six milliliters of completely deaerated ferricyanide-buffer solution are then added to the dilute Hb solution in the Van Croft chamber, and the mixture vacuum extracted by shaking for 6 min. (a longer time than the usual 1–3-min. shaking periods owing to the much larger volumes of solution); the extracted gas is compressed to a volume of about 10 ml. and its $CO_2$ absorbed by admitting 2.5 ml. of deaerated 10% NaOH or 40% NaOH, according to the strength and nature of the buffer used in the ferricyanide-buffer solution, this in turn depending on the buffer content of the dilute Hb solution. Thus, the procedure for various solutions would be as follows.

1. Hb solution in 0.2M $H_3BO_3$ in 0.1M NaOH (pH 9.1): the ferricyanide-buffer solution consists of 1 part of 32% ferricyanide mixed with 5 parts of the strong acetate buffer (see under *Reagents Required*), 40% NaOH then being used for the $CO_2$ absorption.

2. Hb solution in 0.05 M $H_3BO_3$ in 0.025 M NaOH (pH 9.1): 1 part of 32% ferricyanide is mixed with 5 parts of 1 M $KH_2PO_4$, and 10% NaOH is used for the $CO_2$ absorption.

3. Hb solution in 0.05 M phosphate buffer (pH 7.1): 1 part of 32% ferricyanide is diluted with 5 parts of water, and 10% NaOH is used for the $CO_2$ absorption.

In all cases the pH of the mixed Hb-ferricyanide solution in the Van Croft must be not greater than about 7.0 units if the oxygen is to be *completely* extracted. The above ferricyanide-buffer mixtures ensure that this is so in the different Hb solutions which have been studied.

It is obviously vital that the 6-ml. sample of ferricyanide-buffer solution should itself contain no more than 0.1 $\mu$l. dissolved $O_2$ at most. This stringent need is met by evacuating and shaking the ferricyanide-buffer solution in a 120-ml. tonometer with a 30-ml. buret attachment (Fig. 1B), flushing out the gas with 99.7% pure $CO_2$ from a tank and repeating several times, the ferricyanide solution being finally left in the vertical position with a positive pressure of $CO_2$ above it. After flushing the buret lead with a few milliliters of the deaerated ferricyanide solution, 6 ml. of this solution is measured into the Van Croft directly from the buret through mercury with the usual rubber-tip

arrangement. The ferricyanide solution is freshly extracted (as described above) just before each analysis.

The ejection of the whole or an aliquot part of the gas into the Scholander-Roughton syringe capillary calls for no special mention. The volume of Hb solution used in the determination is obtained by ejecting the whole of the solution from the Van Croft chamber into a beaker and weighing it. From the volume so determined 8.5 ml. is subtracted to allow for the 6 ml. ferricyanide solution + 2.5 ml. NaOH solution. The Van Croft chamber is then thoroughly washed, and the top tap regreased, the excess grease being removed by rinsing the bore with acetone, toluene, acetone, and several flushes of water. Water and ether followed by several final water flushes is recommended by Van Slyke and Plazin (*3*), and might be just as (if not more) effective—but the acetone-toluene procedure had already been developed in our laboratory over 10 years ago and has proved quite satisfactory. Some effective method of removing any residual trace of grease is, in any case, essential when working to a precision of $\pm$ 0.1 $\mu$l. gas (S.T.P.).

The accuracy and reliability of the Van Croft procedure can readily be checked by deaerating about 200 ml. of water in a separate tonometer, running about 150 ml. thereof into the Van Croft chamber and vacuum-shaking it therein for 3 successive 6-min. periods, the extracted gas being quantitatively ejected after each shaking. The residual dissolved oxygen in the 150-ml. sample is thereby reduced to less than 0.1 $\mu$l. A small, measured volume of aerated water of known dissolved $O_2$ content is then added to the 150-ml. sample in the Van Croft, mixed therewith and the oxygen content determined as described above. In three recent tests, the following results were obtained:

| | |
|---|---|
| Oxygen found | 4.36, 4.05, 4.13—mean 4.18 $\mu$l. |
| Expected figure | 4.24 $\mu$l. $O_2$ (S.T.P.) |

Similar tests, in which small, measured volumes of aerated water were added to about 150 ml. of completely deoxygenated hemoglobin solution (of the usual dilution), gave equally satisfactory recovery of the added oxygen. This was also the case if the addition of the ferricyanide was delayed for a quarter of an hour, i.e., about the normal time taken to transfer the Hb samples to the Van Croft chamber from the tonometers in which they had been equilibrated, thus showing that no significant oxygen consumption in the unstirred solutions occurs during this time.

### Tonometer Procedure

Volumes of about 250 ml. of dilute Hb in borate- or phosphate-buffer solution are rotated in large evacuated Barcroft tonometers of 2.5- to 3-L. capacity (Fig. 2A), the tonometers having a double-oblique 2-mm.

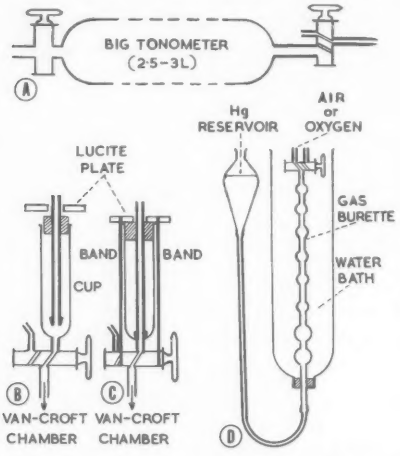


**Fig. 2A.** Large tonometer for equilibration of dilute hemoglobin solutions. **B.** Arrangement for bubbling $CO_2$ through leads from large tonometer. **C.** Method of clamping of connection from large tonometer to base of Van Croft cup. **D.** Mercury reservoir and water-jacketed gas buret with five 10-ml. bulbs and two 25-ml. bulbs.

capillary bore tap at one end and a larger bore single-way tap at the other. Suitable known amounts of oxygen are added to the tonometers to produce the desired final pressures of oxygen at equilibrium. The preparation and loading of the tonometers with solution and gas need meticulous attention, if successful results are to be obtained, and the procedure will therefore be described in full detail.

#### Evacuation of the Tonometers

It is essential that the residual oxygen in the evacuated tonometer, prior to adding the Hb solution and the known amount of oxygen gas, should not exceed 10 $\mu$l. (S.T.P.). This requirement is conveniently met by introducing a few milliliters of water into the tonometer after it has been evacuated in the ordinary way by means of a powerful water-vacuum pump, and then, with the water pump still running, a stream of hot water is sprayed over the outer surface and the tonometer so that the inside water boils rapidly and the stream of hot water vapor effectively sweeps out the last traces of oxygen, as has been proved by control tests. The tonometer is closed off just as the last drop of water disappears from inside and the tonometer taps are both sealed with deaerated water rather than with mercury, which if it later

came in contact with the dilute hemoglobin might inactivate it. The vacuous, water-sealed tonometer is then mounted in a constant-temperature water bath in which it can be rotated for 15-min. equilibration periods.

It is, of course, essential that the tonometer taps should be of best vacuum quality and should be efficiently greased. No leakage of air into the vacuous tonometers can occur while they are immersed in the water-bath: 24-hr. tests with the vacuous tonometers exposed to the atmosphere showed a leakage rate of less than 0.01-mm.-Hg air per hour, which is negligible as regards the present procedure, in which the exposure of the vacuous tonometer to the atmosphere in each experiment, up to the completion of the equilibration, is only of the order of one minute.

#### Loading of Buffer and Hb Solutions into Tonometer in Bath

Usually 240 ml. of aerated or partially aerated buffer solution (the exact volume being known to $\pm$ 0.1 ml.) plus an exactly known volume of the stock reduced Hb solution (close to 10 ml.; the actual volume we have used is 9.66 ml.), is measured into the tonometer in each experiment. Mixtures of (1) 80 ml. aerated buffer plus 160 ml. deaerated buffer, or (2) 120 ml. aerated buffer plus 120 ml. deaerated buffer, or (3) 160 ml. aerated buffer plus 80 ml. deaerated buffer, or (4) 240 ml. aerated buffer plus 0 ml. deaerated buffer provide appropriate amounts of total oxygen to give four points within the range 0–2% $HbO_2$, an allowance being made for the residual $O_2$ content of the 9.66 ml. of reduced Hb solution, which should not exceed 0.002 ml. To obtain points on the equilibrium curve (at 19°) above 2% $HbO_2$, it is necessary to add known supplementary volumes of oxygen gas.

The requisite volumes of buffer solution are placed in 250-ml. tonometers, the stems of which are joined by rubber tube under water to the stem of the double oblique tap of the big tonometer, all air bubbles being carefully removed from the connections. With the lower tap of the buffer solution tonometer fully open the double oblique tap of the big tonometer is cautiously and partially opened so that the buffer solution flows in slowly and smoothly, the time of delivery being about 3 min. It is important to regulate the inflow solely by means of the double oblique tap of the big tonometer, otherwise gas bubbles come off the fluid in the connections between the two tonometers. In the deaerated buffer solution tonometers the gas phase is oxygen-free nitrogen.

The big tonometer is next rotated for 10 min. so as to equilibrate the gas and aqueous phases, and then an exactly known volume (9.66 ml.) of the stock reduced Hb solution is run into the big tonometer, the stock Hb solution being contained in a tonometer having double oblique taps at each end, the volume of which is determined by weighing (9.66 ml.). Nitrogen is used to drive in the Hb solution, which is then followed by 5 ml. of deaerated buffer solution to wash all the traces of hemoglobin in the connections into the big tonometer, which is withdrawn from the bath for a few seconds to mix the Hb and buffer solutions. The tonometer is then returned to the bath and rotated for 15 min., which was found sufficient to establish equilibrium between the dilute Hb solution and the $O_2$ in the gas phase. By this procedure the saturation of the hemoglobin is never allowed to exceed its final equilibrium value and the danger of methemoglobin formation is thereby reduced. At the end of the equilibration period the tonometer is removed from the bath, stood up to drain with the double oblique tap under water and is then moved into position above the Van Croft apparatus.

### Transfer of Equilibrated Hb Solution from Tonometer to Van Croft Apparatus

The tonometer is supported vertically above the Van Croft, with its double oblique tap downwards. To the stem of this tap is attached a short length of glass capillary with a rubber tip at its lower end and, near the top, a rubber stopper with a small rectangular plate of lucite resting on it (Fig. 2B). The upper cup of the Van Croft contains about 1 ml. Hg and several milliliters of water, and the glass capillary with the rubber tip is fitted loosely into the Van Croft cup. The side lead of the double oblique tap of the big tonometer is connected with a reservoir of carbon dioxide gas (99.7% purity) at a slight positive pressure and $CO_2$ is then bubbled gently through the leads and the Van Croft cup for 2 min. so as to clear the connections of all traces of air. The rubber tip is then fitted tightly to the base of the Van Croft cup, the rubber stopper also fitting tightly into the top of the Van Croft cup, and the arrangement strapped securely by two elastic bands around the lucite plate and the top tap of the Van Croft (Fig. 2C). The 8 ml. of completely deaerated solution, which had already been prepared and is waiting in the Van Croft chamber, is then flushed through the capillary tube and the side arm of the double oblique tap of the big tonometer, followed by mercury right up to this tap. The Van Croft mercury reservoir is now lowered so as to apply a vacuum, and a small volume of solution from the big tonometer is drawn down into the

stem, and ejected through the side arm of the double oblique tap. Solution from the big tonometer is then drawn down to the 2-ml. mark of the Van Croft chamber, ejected through the side lead of the double oblique tap of the big tonometer, and the process repeated a second time. By this procedure all the connections between the big tonometer and the Van Croft chamber are completely freed of air bubbles and of aerated fluid, and the main sample of equilibrated Hb solution is then drawn down slowly into the Van Croft chamber without any contamination by extraneous oxygen. The delivery of the main sample should be very slow and regular, taking in all about 8 min. This is conveniently achieved by attaching the mercury reservoir of the Van Croft to a metal platform which is geared to descend at a rate of about 1.2 cm./min. When about 150 ml. of Hb solution has been drawn into the Van Croft chamber (as indicated by a mark thereon), the delivery is stopped and, after checking by eye that no gas bubbles whatever are present in the chamber, the remainder of the analysis is completed as described above.

For results of maximum accuracy, the Van Croft chamber should be maintained at constant temperature by circulating water from a thermostatically controlled water-bath through the jacket surrounding the chamber.

The Van Croft and tonometer procedures may sound formidable and complicated as a whole, though actually none of the individual steps is difficult. Patience, care, and concentration are certainly required but, given these attributes, the technic should be within the range of any research worker who is already well-trained in Van Slyke gasometric methods. With the aid of a competent technical assistant, it is possible to obtain 3, or at most 4, equilibrium points in the course of one working day, since the procedure for each point takes 2 hr. or a little over.*

### Typical Calculation

Fluid introduced into big tonometer

| | |
|---|---|
| Aerated 2% borax ($Na_2B_4O_7$, 10 $H_2O$) | 120.00 ml. |
| Deaerated 2% borax | 120.00 ml. |
| Stock reduced Hb solution | 9.66 ml. |
| Deaerated 2% borax to wash in Hb solution | 5.00 ml. |
| | 254.7 ml. |

*My warmest thanks are due to my personal assistant, Mr. K. A. Edwards, for the unremitting and meticulous help he has given me in upwards of 200 Van Croft determinations.

Oxygen introduced into big tonometer

$= 120 \times 0.00571$ (i.e., measured $O_2$ content of aerated 2% borax in ml. $O_2$ S.T.P. per ml. solution)

$= 0.685$ ml.

Van Croft Analysis

$p_{1.5} = 231.5$

at 21°

$$p_{2.5} = \frac{218.5}{13.0}$$

Percentage oxygen in gas bubble (by syringe analysis) = 48.5

Volume of $O_2$ (S.T.P.) extracted in Van Croft chamber

$$= \frac{13.0 \text{ (i.e., } p_{1.5} - p_{2.5})}{760 \text{ mm. Hg}} \times 0.48 \text{ (calibrated ''0.5'' ml. volume of Van Croft chamber}$$

$\times$ 48.5% (% $O_2$ in bubble) $\times$ 273/(273 + 21) (correction to 0°)

$= 0.00382$ ml.

Volume of Hb sample taken into Van Croft chamber = 152 ml.
Residual $O_2$ content of fluid in tonometer at end of equilibration

$= 254.7 \times 0.00382/152 = 0.0064$ ml.

Oxygen in gas phase of tonometer at end of equilibration

$= 0.685$ (total $O_2$ introduced) $- 0.0064 = 0.679$ ml.

Volume of tonometer = 2894 ml.

Volume of gas phase in tonometer $= 2894 - 254.7 = 2639$ ml.

Oxygen pressure in tonometer at end of equilibration at 19°

$$= \frac{0.679 \times 760 \times (273 + 19)}{2639 \times 273} = 0.2095 \text{ mm. Hg}$$

Dissolved $O_2$ per 100 ml. of equilibrated solution in tonometer

$$= \frac{0.2095}{760} \times 2.93 \text{ (solubility coefficient of } O_2 \text{ in 100 ml.)}$$

$= 0.000805$ ml.

Total $O_2$ (dissolved and combined) in 100 ml. of equilibrated solution

$$= \frac{0.00382 \times 100}{152} = 0.002518 \text{ ml.}$$

Combined $O_2$ in 100 ml. of equilibrated solution

$$= 0.002518 - 0.000805 = 0.00171 \text{ ml.}$$

Gas-combining capacity of 100 ml. of equilibrated solution

$$= 0.152 \text{ ml.}$$

$$\% \text{ HbO}_2 = 100 \times 0.00171/0.152 = 1.13$$

$$p\text{O}_2 = 0.2095 \text{ mm. Hg}$$

No allowance has been made in the above calculation for the residual $O_2$ content of the stock reduced Hb solution, since the amount of the latter (about 0.006 ml. $O_2$) was less than 1% of the $O_2$ introduced via the aerated borax solution (0.685 ml.).

### Accuracy and Reliability of Combined Van Croft-Tonometer Procedure with Low Amounts of $O_2$

A convenient and searching test of the method is to carry through the whole procedure with aerated buffer solution alone, but without any added Hb solution, and then to compare the observed $O_2$ content of the final Van Croft sample with that calculated in the manner just described—the $O_2$ content of the aerated buffer having been independently determined by ordinary Van Slyke gasometric technic. The following results for the $O_2$ content in $\mu$l. $O_2$ (S.T.P.) per 150-ml. sample were obtained in a series of such tests, the theoretical figure being shown in parentheses: 2.79 (2.86), 2.85 (2.86), 2.68 (2.76), 2.76 (2.66), 2.57 (2.66). The maximum discrepancy is seen to be $\pm$ 0.1 $\mu$l.: this corresponds to an accuracy of $\pm$ 0.033% $HbO_2$ in samples of hemoglobin containing 0.15 gm. Hb/100 ml., i.e., the average concentration used in the present work. Such accuracy, with actual Hb solutions of this dilution, was in fact attained as shown by (1) the agreement between duplicates, and by (2) the observed linear relation between % $HbO_2$ and equilibrium $pO_2$ in the range 0–2% $HbO_2$. In both cases the average discrepancy was found to be $\pm$ 0.033 % $HbO_2$.

### Gasometric Determination of $O_2$ Equilibrium Curve of Dilute Sheep Hb Solutions over Main Part of Curve (5–95% $HbO_2$)

The procedure is identical with that which has just been described at length for the bottom of the oxygen-equilibrium curve, except for the following additional features.

Accurately known volumes of oxygen gas have also to be added to the tonometers after the buffer solutions and the Hb solutions have been introduced therein. These required volumes are measured from a specially constructed gas buret with attached mercury reservoir. The gas buret (Fig. 2D) is made up of 7 bulbs (five of about 10-ml. capacity each, and two of about 25-ml. capacity each—the exact volumes are determined by calibration with mercury) and has a double oblique tap at the top. The buret is immersed in a well-stirred water jacket of the same temperature as that of the tonometer bath. At atmospheric pressure, it is filled down to the appropriate bulb with outside air (20.93% $O_2$) or with tank oxygen (99.8% purity). The inside of the buret is kept moist by occasionally adding small amounts of water so that the gas added to the tonometer is always saturated with aqueous vapor. The gas buret is connected to the tonometer in the bath by a length of glass capillary tubing of measured volume (1.63 ml.), and allowance is made for the $O_2$ content of the gas in this connection, the whole of it being admitted into the tonometer in the bath. All rubber joints between gas buret and the bath tonometer are surrounded by water to prevent leakage of air.

The volume of residual gas in the Van Croft chamber, after the absorption of the $CO_2$ by running in 10% (or 40%) NaOH, may be too large for it to be possible to wash the top part of the chamber with the mixed solution. Appreciable films of unmixed 10% (or 40%) NaOH may thus remain at the top, and cause vapor pressure errors. To eliminate this well-known source of possible trouble, the 2.5 ml. of 10% (or 40%) NaOH should be followed by 1.0 ml. of deaerated 1% NaOH before the $p_{1.5}$ reading is taken.

The per cent oxygen in the residual gas, after the absorption of the $CO_2$, rises corresponding with the per cent $HbO_2$ in the sample to be analyzed, and may go as high as 99%. The ordinary Scholander-Roughton syringe-capillary is only capable of analyzing the per cent oxygen in the range 90–100% to within about ± 0.3% at best—which is just, but not more than just, exact enough to give the desired accuracy in the % $HbO_2$ estimation when it is above 75%. In a modified form of the syringe-capillary, which I have designed especially for estimating small amounts of residual gas impurities to within ± 0.05% (i.e., about six times better), the terminal cup of the ordinary Scholander-Roughton apparatus (Fig. 3A) is replaced by a 16-cm. length of glass capillary tubing of about 1.5 mm. internal diameter. This is bent through two right angles, the last 8 cm. being straight and ending in a shape

suitable to be fitted with the usual kind of rubber tip (Fig. 3B). With the gas in the chamber compressed and the tip seated firmly in the cup, a sample of gas can be drawn into the syringe barrel, previously filled with bubble-free 10% NaOH or 30% NaCl solution. The syringe is

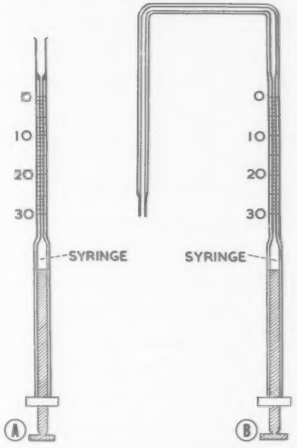


**Fig. 3A.** Ordinary Scholander-Roughton syringe capillary (9). **B.** Modified Scholander-Roughton syringe capillary for accurate analysis of percentage impurity in gases at least 90% pure.

then lifted from the cup, the rubber tip removed, and the end immersed in NaOH (or NaCl) solution. By manipulating the plunger of the syringe, the volume of the gas sample is reduced so that the gas sample just fills the bore of the tube from the end to the last graduation on the capillary. This volume of gas is then sealed by drawing up a few millimeters of fluid, and the end then immersed in a beaker containing oxygen-absorbent solution. This is drawn up in the usual way, and the residual nitrogen is finally measured in the graduated capillary of the syringe, each division of which in the present apparatus corresponds to 0.429% of impurity in the original gas sample. A small blank correction of 0.16% has to be subtracted from the impurity percentage to allow (probably) for diffusion of a small amount of dissolved nitrogen into the gas sample from the oxygen absorbent whilst the oxygen absorption is taking place.*

## Experimental Results

Adair's theory (*4*) that the combination of hemoglobin with oxygen takes place in four successive stages—$Hb_4+O_2 \rightleftharpoons Hb_4O_2$; $Hb_4O_2+O_2$

*My thanks are due Dr. R. L. J. Lyster for providing me with some of the details in the use of this modified syringe capillary.

$\rightleftharpoons Hb_4O_4$; $Hb_4O_4+O_2 \rightleftharpoons Hb_4O_6$; $Hb_4O_6+O_2 \rightleftharpoons Hb_4O_8$—led him to the following equation for the equilibrium between oxygen and *ideal solutions* of hemoglobin (an "ideal" solution is one in which the osmotic pressure is proportional to the concentration)

$$\frac{y}{100} = \frac{A_1p + 2\,A_2p^2 + 3\,A_3p^3 + 4\,A_4p^4}{4\,(1 + A_1p + A_2p^2 + A_3p^3 + A_4p^4)} \qquad (1)$$

where $y$ is the oxygen percentage saturation (% $HbO_2$), $p$ is the oxygen pressure, and $A_1$, $A_2$, $A_3$, $A_4$ are constants which are functions of the several equilibrium constants of the intermediate reactions ($Hb_4 + O_2 \rightleftharpoons Hb_4O_2$, etc.).

Equation 1 is valid, whether the four heme groups in the hemoglobin molecule ($Hb_4$) are identical, or differ in their reactivity towards oxygen. Adair showed that hemoglobin does behave "ideally" in solution at concentrations less than about 4 gm. Hb/100 ml., but that in the red cell, where the concentration is around 30 gm. Hb/100 ml., there is about a fourfold deviation from the ideal. Modifications of Equation 1 may therefore be necessary before it can be properly applied to whole blood. In spite of this, there have been numerous recent attempts to test Equation 1 on the classical data of Dill (*11*) on the $O_2$-Hb equilibrium in whole human blood at pH 7.4, 38°, and at a constant pressure of carbon dioxide (40 mm. Hg). Dill's results, though admirable for their original physiological purpose, are not, however, necessarily suitable for test by Equation 1, in view of the nonideality of the hemoglobin in the red cell. There are also other physico-chemical uncertainties about the Dill data, one of which is the lack of any correction for the varying amount of carbon dioxide bound to the hemoglobin in the carbamino form, in different regions of the equilibrium curve.

### Results at pH 9.1

Forbes and Roughton (*12*) seem to have been the first to make accurate gasometric determinations of the $O_2$-Hb equilibrium curve in "ideal" hemoglobin solutions, the concentrations they used being around 0.4 gm. Hb/100 ml. Most of the experiments were done at 19° in 0.2M borate buffer, pH 9.1. In this alkaline range the $O_2$-Hb equilibrium is not sensitive to small changes in pH and the borate acts as quite a good preservative. As already mentioned, sheep hemoglobin has no tendency to split into half-molecules under such conditions.

The open circles in Fig. 4 are plotted from the composite data of Forbes and Roughton at pH 9.1, 19° as summarized by Roughton (*13*);

the actual position, but not the *shape,* of the equilibrium curve varied slightly from one sheep sample to another, so the results were all normalized to an oxygen pressure of 3 mm. Hg at 50% $HbO_2$ by multiplying the pressures by an appropriate factor in the case of each individual sheep Hb sample. Thus, if in any particular Hb sample it was found that $p_{50}$, (the $pO_2$ for 50% $HbO_2$) equaled 2.5 mm. Hg, all the oxygen pressures at different parts of the equilibrium curve for that particular sample were multiplied by 3.0 mm./2.5 mm. (i.e., by 1.2) before being plotted.

The closed circles, the vertical crosses, and the oblique crosses in Fig. 4 show results by the present technic over the middle of the equilibrium curve for dilute solutions of Hb from three separate sheep, each of genetic type A, the oxygen pressures all being normalized to a $p_{50}$ of 3 mm. Hg in each case. The actual oxygen pressures were, in point of fact, multiplied by the same factor—1.06—in all three cases, the individual points without "normalization" all falling within experimental error on the same curve. The agreement with the 30-year-old data of Forbes and Roughton is very satisfactory and well within the rather larger experimental error they recorded (1–1.5% $HbO_2$). At the time of their work the two genetic types of sheep blood had not

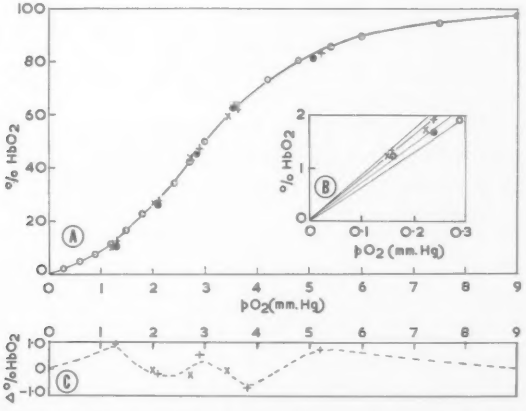


**Fig. 4A.** Equilibrium curves for dilute sheep Hb solutions in 0.2 M borate buffer, pH 9.1 at 19°. Data of Forbes and Roughton (*12*) normalized to $P_{50} = 3$ mm. Hg. Data for hemoglobin from three separate sheep (genetic type A) by present technic. **B.** Magnified view of bottom of equilibrium curves for sheep hemoglobins used in Fig. 4A. **C.** Discrepancy (△ % $HbO_2$) between observed and calculated equilibrium results for two of sheep hemoglobins used in Fig. 4A.

been recognized and the hemoglobin used by them might have been sheep Hb A, sheep Hb B (the electrophoretically slow type), or mixtures of sheep Hb A and B. Probably, however, their hemoglobins were mainly of type A, since the average $p_{50}$ for their Hb samples was only about 6% higher than the average $p_{50}$ for the three recent samples of Hb A plotted in Fig. 4, whereas the average $p_{50}$ for sheep Hb B is about 25% higher.

The inset in Fig. 4B shows the especially accurate results for the three recent Hb samples in the range 0–2% $HbO_2$. The equilibrium curve is linear to within ± 0.05% $HbO_2$, as would be expected from the limiting form of equation (1) at low values of p, viz.,

$$\frac{y}{100} = \frac{A_1 p}{4} \tag{2}$$

The value of $A_1$ is thus obtainable independently, leaving only three disposable constants, $A_2$, $A_3$, and $A_4$, in Equation 1. The actual values of $A_1$ for the three Hb samples are 0.292 (± 0.014), 0.326 (± 0.0095), and 0.349 (± 0.0070), respectively. Such individual variations in sheep hemoglobins of one genetic type are within the range previously reported by Kernohan (*14*) for $A_1$ in the case of 25-times-stronger solutions (4 gm. Hb/100 ml.) For the old Forbes-Roughton data the value for $A_1$, based on the oxygen pressure at 2% $HbO_2$, is 0.26 ± about 0.1 $(mm.\ Hg)^{-1}$, the uncertainty in the actual figure being around 10 times greater than in the present especially accurate work at the bottom of the oxygen-equilibrium curve.

In order to test Adair's equation critically it is also necessary, as Roughton, Otis and Lyster (*15*) showed, to have results of special accuracy (± 0.05% $HbO_2$) at the top of the curve, where the equation tends to the limiting form

$$\frac{y}{100} = 1 - \frac{A_3}{4\ A_4\ p} \tag{3}$$

If the value of $A_4/A_3$ can thus be independently determined the number of disposable constants are then in effect reduced to two. Roughton (*5*) has described a successful method for obtaining results accurate to ± 0.05% $HbO_2$ at the top of the curve but his procedure is so far available only at alkaline pH 9.1 and for hemoglobin concentrations of 4 gm./100 ml. or higher. It is particularly unfortunate that it cannot be used in the physiological pH range (7.0–7.4). There is, however, an alternative, though indirect, way of obtaining $A_4/A_3$ for dilute Hb so-

lutions, which can be used at any pH over the range 6-9. It is assumed that

$$\frac{A_4}{A_3} = K_4 \text{ (the equilibrium constant of the reaction } Hb_4O_6+O_2 = Hb_4O_8)$$

$$= \frac{k_4' \text{ (the velocity constant of the reaction } Hb_4O_6+O_2 \rightarrow Hb_4O_8)}{k_4 \text{ (the velocity constant of the reaction } Hb_4O_8 \rightarrow Hb_4O_6+O_2)}$$

The velocity constants $k_4$ and $k_4'$ are obtainable separately by the methods of Gibson and Roughton (*2, 5, 16*) and on this basis it is found that for dilute sheep Hb *A* solution at pH 9.1, 0.2 M borate, 19°

$$\frac{k_4'}{k_4} = K_4 = 2 \pm 0.2 \text{ (mm. Hg)}^{-1}$$

This value of $K_4$ is about the same as the average value of Roughton *et al.* (*15*) in stronger solution (4 gm. Hb/100 ml.) in contradistinction to $A_1$ which is, according to Roughton *et al.* (*15*, Fig. 5), 2–3 times greater in the dilute than in the stronger sheep Hb solutions.

At pH 7.1, 0.05 M phosphate buffer, 19°, the value of $K_4$ for dilute sheep Hb solution (0.15 gm./100 ml.) is found, by the same procedure, to be $1.6 \pm 0.2$ (mm. Hg)$^{-1}$. The effect of pH on $K_4$, over the range 7.1 to 9.1, is thus only slight and contrasts very markedly with its effect on $p_{50}$, which at pH 7.1 is about 4 times greater than at pH 9.1.

With the values of $A_1$ and $A_4/A_3$ already settled, or approximately so, it is then possible to estimate by means of the least-squares statistical procedure described by Daniels in the paper of Roughton *et al.* (*15*) the best values of $A_1$, $A_2$, $A_3$, and $A_4$ with relatively small standard errors. On the other hand, with data of only the customary accuracy (± 1% $HbO_2$) over the main part of the equilibrium curve (10–95% $HbO_2$), but without special information as to $A_1$, and $A_4/A_3$, the estimation of $A_2$ and/or $A_3$ would, as Roughton *et al.* (*15*) have shown, be subject to unfeasibly large standard errors.

More recently P. Blundell of the Department of Applied Mathematics of the University of Sheffield has set up an automatic electronic program for computing the constants $A_1$, $A_2$, $A_3$, and $A_4$, together with their standard errors and the discrepancies of the calculations from the observed results. Each such computation takes only about 7 min. of machine time.*

Table 1 shows the computed values of $A_1$, $A_2$, and $A_3$, with their estimated standard errors underneath in parentheses, for two of the Hb

*I am deeply indebted to Mr. Blundell for this most useful contribution to our work.

**Table 1.** COMPUTED VALUES OF $A_1$, $A_2$, AND $A_3$ FOR DILUTE Hb SOLUTIONS AT pH 9.1, 19°, WITH 3 ASSUMED VALUES FOR $A_4/A_3$

| | *Sheep A 38* | | | *Sheep A 44* | | |
|---|---|---|---|---|---|---|
| $A_4/A_3$ | 1.6 | 2.0 | 2.4 | 1.6 | 2.0 | 2.4 |
| $A_1$ | 0.32762 | 0.32574 (±0.00950) | 0.32445 | 0.29426 | 0.29187 (±0.01390) | 0.29023 |
| $A_2$ | 0.03204 | 0.03728 (±0.01070) | 0.04087 | 0.02255 | 0.02829 (±0.00950) | 0.03222 |
| $A_3$ | 0.01226 | 0.00998 (±0.00019) | 0.00841 | 0.01186 | 0.00963 (±0.00025) | 0.00811 |

samples plotted in Fig. 4, on the assumption that $A_4/A_3 = 2$, together with the effect of ± 20% variations in the assumed value of $A_4/A_3$. Figure 4C gives the discrepancies in per cent $HbO_2$ between the observed and the computed values for the case of $A_4/A_3 = 2$. The mean discrepancy (disregarding signs), is 0.44% $HbO_2$; regarding the signs, it is + 0.098% $HbO_2$. Both these figures are satisfactorily small.

If, as was formerly postulated (*17*), the four hemes in hemoglobin are equivalent in their reactivity to small ligand molecules such as oxygen, it would follow that $A_1 = K_1$, $A_2/A_1 = K_2$, $A_3/A_2 = K_3$, and $A_4/A_3 = K_4$, where $K_1$, $K_2$, $K_3$, and $K_4$ are the respective equilibrium constants of the reactions $Hb_4+O_2 \rightleftharpoons Hb_4O_2$, $Hb_4O_2+O_2 \rightleftharpoons Hb_4O_4$, $Hb_4O_4+O_2 \rightleftharpoons Hb_4O_6$, and $Hb_4O_6+O_2 \rightleftharpoons Hb_4O_8$. Although recent work on the amino-acid sequence and X-ray analysis of hemoglobin has shown that the chemical environment of the four heme groups in the hemoglobin molecule is not identical, and hence that they are not necessarily equivalent in their reactivity with oxygen, it is nevertheless worthwhile to calculate the values of $K_1$, $K_2$, $K_3$, and $K_4$ on the old simple equivalence principle. The results for the data of Table 1, with $A_4/A_3 = 2.0$, are shown in Table 2, which also gives the values of the ratios $r_2 = K_2/K_1$; $r_3 = K_3/K_1$; and $r_4 = K_4/K_1$.

If the heme groups are not only equivalent but also independent of one another, the values of the ratio $r_2$, $r_3$, and $r_4$ should be determined by simple statistical principles and should be equal to $3/8$, $1/6$, and $\frac{1}{16}$, respectively, as may be seen by comparing, e.g., the first intermediate reaction ($Hb_4+O_2 \rightleftharpoons Hb_4O_2$) and the second intermediate reaction ($Hb_4O_2+O_2 \rightleftharpoons Hb_4O_4$). In the first case, the chance of an oxygen molecule combining with hemoglobin (i.e., the velocity constant of the reaction $Hb_4+O_2 \rightarrow Hb_4O_2$), should be simply proportional to the number of uncombined heme groups therein, viz., 4, whereas the chance of an oxygen molecule dissociating (i.e., the velocity constant of the reaction

**Table 2.** COMPUTED VALUES OF $K_1$, $K_2$, $K_3$, AND $K_4$ AND OF THE RATIOS $r_2$ ($= K_2/K_1$), $r_3$ ($= K_3/K_1$), AND $r_4$ ($= K_4/K_1$)*

| *Sheep A 38* | | *Sheep A 44* | | |
|---|---|---|---|---|
| $K_1$ 0.3257 | | $K_1$ 0.2919 | | |
| | $r_2$ 0.351 | | $r_2$ 0.332 | mean $r_2$ 0.341 |
| $K_2$ 0.1144 | | $K_2$ 0.0969 | | |
| | $r_3$ 0.822 | | $r_3$ 1.177 | mean $r_3$ 1.000 |
| $K_3$ 0.2677 | | $K_3$ 0.3405 | | |
| | $r_4$ 6.14 | | $r_4$ 6.85 | mean $r_4$ 6.50 |
| $K_4$ 2.0 | | $K_4$ 2.0 | | |

*For data of Table 1, with $A_4/A_3 = 2$.

$Hb_4O_2 \rightarrow Hb_4 + O_2$), should conversely be proportional to the number of combined heme groups, viz., 1. The equilibrium constant ($K_1$), being the ratio of the velocity constants, would thus be proportional to 4/1. Similarly, the value of $K_2$ should be proportional to 3/2, so that $r_2 = K_2/K_1 = 3/2 \div 4/1 = 3/8$. A similar extension to the third and fourth intermediate reactions gives $r_3 = 1/6$ and $r_4 = \frac{1}{16}$.

The mean values of $r_2$, $r_3$, and $r_4$ for the two cases in Tables 1 and 2 are seen to be 0.341, 1.000, and 6.50, respectively. The value of $r_2$ is thus close to the "statistical" figure (3/8 or 0.375), but the value of $r_3$ is $1.000 \div 1/6$, i.e., 6 times, and the value of $r_4$ is $6.5 \div \frac{1}{16}$, i.e., 104 times greater than the respective statistical figures. Discrepancies of this kind are customarily attributed to *interaction* between the heme groups: the suggestion from the present approach is that in dilute sheep Hb solutions in borate buffer at pH 9.1, 19°, interaction does not develop until the third oxygen molecule is combining and increases more markedly still at the fourth and final stage.

### Results at pH 7.4

Table 3 shows similar data on two dilute sheep Hb *A* (about 0.15 gm./100 ml.) solutions at pH 7.1, 0.05 M phosphate buffer, and 19°, one of the animals from which the blood was drawn being the same as in Table 1 at pH 9.1. The ionic strength I* of 0.05 M phosphate, pH 7.1, = 0.1 and is the same as that of the 0.2 M borate buffer pH 9.1 used in Table 1. The value of $A_1$ at pH 7.1 is about one quarter that of $A_1$ at pH 9.1, and the mean values of the ratios $r_2$, $r_3$, and $r_4$ are 0.36, 0.33, and 21.8. The value of $r_2$ is again close to the "statistical" figure (0.375)

*The ionic strength of a solution $= I = \frac{1}{2} \Sigma C_m V_m^2$, where $C_m$ is the concentration of an ion of species m, $V_m$ is the valency of the ion of species m, and $\Sigma$ is the sum of the terms $C_m V_m^2$ for all the different ion species present in the solution.

**Table 3.** COMPUTED VALUES OF $A_1$, $A_2$ AND $A_3$ FOR DILUTE Hb SOLUTIONS AT pH 7.1, 19°*

| *Sheep A 38* | | | *Sheep F 22* | | | |
|---|---|---|---|---|---|---|
| $A_1$ 0.08355 | $K_1$ 0.08355 | | $A_1$ 0.06545 | $K_1$ 0.06545 | | |
| | | $r_2$ 0.195 | | $r_2$ 0.519 | mean $r_2$ | 0.355 |
| $A_2$ 0.001363 | $K_2$ 0.01632 | | $A_2$ 0.00223 | $K_2$ 0.03408 | | |
| | | $r_3$ 0.362 | | $r_3$ 0.302 | mean $r_3$ | 0.332 |
| $A_3$ 0.000041 | $K_3$ 0.03024 | | $A_3$ 0.000044 | $K_3$ 0.01977 | | |
| | | $r_4$ 19.140 | | $r_4$ 24.43 | mean $r_4$ | 21.78 |
| $A_4$ 0.0000650 | $K_4$ 1.6 | | $A_4$ 0.0000704 | $K_4$ 1.6 | | |

*$A_4/A_3$ assumed equal to 1.6; computed values of $K_1$, $K_2$, $K_3$, $K_4$ and of $r_2$, $r_3$, $r_4$ also given.

but the value of $r_3$ is now only twice the statistical figure instead of 6 times as at pH 9.1. On the other hand, the value of $r_4$ at pH 7.1 is 349 times the statistical figure, as compared with the more than hundred-fold effect at pH 9.1. The results at pH 7.1 thus differ from those at pH 9.1 in suggesting only about one third as much interaction at the stage of the third intermediate reaction, but on the other hand they indicate 3 to 4 times greater interaction at the fourth and final intermediate reaction.

Thus the effect of pH over the range 7.1–9.1 on the several intermediate reactions is not the same, in spite of the fact that the equilibrium curves over the main range (10–90% $HbO_2$) can be made nearly, though *not quite exactly,* to coincide if the oxygen pressures at pH 7.1 are all divided by a constant factor of 4.08. There has been a tendency on the part of some writers to assume that if the $O_2$-Hb equilibrium curves at two different pH values can be made to coincide by changing the oxygen pressures by a factor of $\phi$, then each individual equilibrium constant of the intermediary series must differ by a factor of $\phi$;

$$\frac{A_1'}{A_1} : \frac{A_2'}{A_2} : \frac{A_3'}{A_3} : \frac{A_4'}{A_4} :: \phi : \phi^2 : \phi^3 : \phi^4$$

where symbols without primes refer to one pH and symbols with primes refer to the other pH. With $\phi = 4.08$, this relationship would give:

$$\frac{A_1'}{A_1} : \frac{A_2'}{A_2} : \frac{A_3'}{A_3} : \frac{A_4'}{A_4} :: 4.08 : 16.6 : 67.9 : 277.1$$

as compared with the observed ratios at pH 7.1 and pH 9.1 (Tables 1–3) of:

$$\frac{A_1'}{A_2'} : \frac{A_2'}{A_2} : \frac{A_3'}{A_3} : \frac{A_4'}{A_4} :: 4.08 : 16.7 : 254 : 287$$

Three of the ratios conform to the simple hypothesis, but there is a major break-down as regards $A_3'/A_3$.

A similar admonition in regard to the effect of temperature was long ago lodged by Roughton (*18*) and was validated later by the experiments of Roughton *et al.* (*15*, Fig. 2) on sheep hemoglobin solutions at pH 9.1, at 0.2° and at 19°. Over the main range of the equilibrium curves, the points at 0.2° could be made to coincide, within experimental error, with the points at 19° by multiplying all the oxygen pressures at 0.2° by a constant factor of 3.737. Nevertheless, the ratio of $A_1$ at 0.2° to $A_1$ at 19°, was found to be 7, and not 3.737.

In discussing their results on the $O_2$-Hb equilibrium in dilute sheep Hb solutions, Forbes and Roughton (*12*) pointed out over 30 years ago that one (*but by no means the only*) way of accounting for such findings on the basis of the general Adair equation, was to assume that $A_1$, $A_2$, and $A_3$ were statistically interrelated. Roughton (*18*) found that his data on strong solutions of bovine hemoglobin could also be closely fitted in this way, and later (*13*, in equation A) gave, for this special case, a simplified form of the general Adair equation, containing only two disposable constants. He stressed again, however, that the simplified equation was only one of many ways of accounting for the results. Quite recently Margaria and his colleagues (*19*) have applied the same treatment to the $O_2$-Hb equilibrium in whole blood under physiological conditions, e.g., to the Dill data (*11*). In unpublished calculations on the latter I have found, however, that if the assumed ratio of $A_4$ to $A_3$ is varied fivefold, or even tenfold, the observed and calculated results can still be made to agree, within experimental error, by making relatively small adjustments in the absolute value of $A_4$. This is possible because of the dominant influence of the $A_4p^4$ of the Adair equation, over the main range of the equilibrium curve. Independent information as to the value of $A_4/A_3$ is thus, as has already been stressed, quite indispensable: when this is supplied it is found, in the case of the dilute sheep Hb solutions, that the statistical principle only applies to $A_1$ and $A_2$, but breaks down as regards $A_3$. It remains to be seen what will happen, if and when such information is available in the case of human whole blood.

### Possible Extension to More Physiological Conditions

The theoretical propriety, from the physico-chemical viewpoint, of applying Adair's equation in its unmodified form (1) to data on whole blood at constant $CO_2$ pressure has already been questioned earlier in

this paper. There is also the statistical difficulty that, up to the present, there do not appear to exist for whole blood any data of specially high accuracy ($\pm$ 0.05% $HbO_2$) at either of the two critical regions of the $O_2$-Hb equilibrium curve (the extreme top and the extreme bottom). For a reasonably satisfactory statistical analysis such data, as already stressed, are necessary in *both* regions (*15*). There seems no apparent reason why a simple modification of the method described in this paper should not be applied to whole blood so as to give an accuracy of $\pm$ 0.05% $HbO_2$ in the range 0–2% $HbO_2$, and it is hoped before long to make trials along these lines. Specially accurate data at the top of the equilibrium curve cannot, however, be obtained for whole blood at physiological pH either by Roughton's method (*5*) or by the indirect, kinetic approach described in the present paper. It is hoped, instead, to apply to this problem an oxygen electrode technic along the lines already developed by Glauser (*20*).

In the meanwhile it is of interest to note that—if the oxygen pressures noted by Bartels and Harms (*21*) in their work on the oxygen equilibrium curve for whole sheep blood at pH 7.2 and 37° are divided by a factor of 3.554—the points obtained fall within experimental error ($\pm$ 1% $HbO_2$) on the same curve as do our own data on dilute sheep Hb solution at pH 7.1 and 19° (Fig. 5). It might again be tempting to assume therefrom that $A_1 = 3.554 \times A_1'$; $A_2 = 3.554^2 \times A_2'$; $A_3 = 3.554^3 \times A_3'$; and $A_4 = 3.554^4 \times A_4'$, where symbols without primes refer to the dilute Hb solution at 19° and symbols with primes refer to the whole blood at 37°. The instances given above in regard to the effect of pH and temperature, should, however, provide a sufficient deterrent against making such assumptions in any comparison of whole blood and dilute Hb solution, and they do indeed reinforce the desirability of obtaining especially accurate points at the two ends of the $O_2$-Hb equilibrium for whole blood.

#### Hill's Equation for the $O_2$-Hb Equilibrium Curve

In recent years reports (*17, 22–24*) have indicated renewed interest in the use of Hill's empiric equation

$$\frac{y\ (\%\ HbO_2)}{100} = \frac{K\ p^n\ (\text{oxygen pressure})}{1 + K\ p^n} \qquad (4)$$

for quantitative characterization of the $O_2$-Hb equilibrium. Any possible theoretical basis for this equation disappeared long ago when Adair (*4*) discovered that the molecule of hemoglobin contains 4 atoms

of iron, whereas the values of $n$ required to fit the equilibrium curves range from 1.5 to 3. Furthermore, $n$ is not constant over the whole curve, for at the top and bottom of the curve $n$ tends to unity in accordance with the limiting forms of Adair's equation—see Equations 2 and

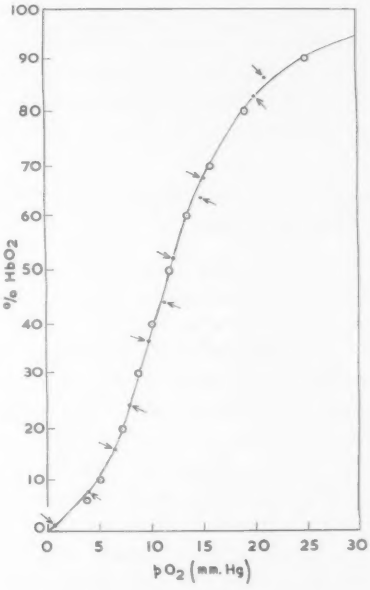


**Fig. 5.** Oxygen equilibrium curves for whole sheep blood (*21*) and dilute sheep Hb solutions by present technic. Circles indicate Bartels and Harms data on whole blood at pH 7.2, 37°, with oxygen pressures all divided by factor 3.554. Arrows indicate sheep Hb A solutions in 0.05 M phosphate, pH 7.1 at 19°.

3 above—although over the main range of the curve, $n$ is empirically (and rather surprisingly) constant or very nearly so for any given buffer solution, and is only slightly (*22*), if at all (*24*), affected by pH. For the data of the present paper over the middle of the curve, $n = 2.9$ for pH 9.1, 0.2 M borate buffer; and $n = 2.8$ for pH 7.1, 0.1 M phosphate buffer.

If the buffer concentration and/or the neutral salt concentration is varied, there is a significant effect on $n$, the value of which rises from about 1.5 at very low ionic strength to around 3 at $I = 1$ (*23*). There is indeed an approximately linear relation between $n$ and log (buffer concentration) or log (salt concentration). So far, only preliminary studies have been made by the present methods on the effect of varying buffer and/or neutral salt concentration upon the relative values of $A_1$, $A_2$, $A_3$, and $A_4$, and there is an obvious field here for further work.

There has been a tendency to regard $n$ as a measure of the degree of interaction—thus, if $n = 1$ there is no interaction, whereas if $n = 4$ there is maximal interaction. Values of $n$ between 1 and 4 correspond to intermediate degrees of interaction. This line of approach is attractively simple and convenient from the purely empiric and phenomenologic viewpoints, but it has the disadvantage that it tends to obscure considerable detailed differences in interaction which may occur without there necessarily being any significant change in n. For this reason the present writer fears that it may inhibit advances in our knowledge of this entrancingly interesting and biologically fundamental equilibrium.

### Correlation of Equilibrium and Kinetic Data on $O_2$-Hb Reactions in Dilute Solutions of Sheep Hb

Q. H. Gibson and I have for several years been working at the kinetics of the four-stage reversible reactions of oxygen with hemoglobin. Even on the simple "equivalent-heme" hypothesis, there would be a total of eight separate velocity constants involved, viz., four combination velocity constants: $k_1'$, $k_2'$, $k_3'$, and $k_4'$, and four dissociation velocity constants $k_1$, $k_2$, $k_3$, and $k_4$, but related to the equilibrium constants by the equations $k_1'/k_1 = K_1$; $k_2'/k_2 = K_2$; $k_3'/k_3 = K_3$; and $k_4'/k_4 = K_4$. The determination, by the methods given in this report, of the four equilibrium constants would thus reduce the eight disposable velocity constants in effect to four. Furthermore, $k_4$, the velocity constant of the reaction $Hb_4O_8 \rightarrow Hb_4O_6 + O_2$, and possibly also $k_1'$, the velocity constant of the reaction $Hb_4 + O_2 \rightarrow Hb_4O_2$, can be separately determined (*2*, *16*), leaving then only two disposable velocity constants. During the next two years Gibson and I hope to see how far along these lines it may be possible, as a first approximation, to describe the kinetics of combination of oxygen with sheep hemoglobin. The work described in the present paper is certainly an indispensable prelude thereto.

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# Hemoglobin at High Altitude as Related to Age

*D. B. Dill, J. W. Terman, and F. G. Hall*

**During the summer of 1962 the early phase of acclimatization to high altitude was studied in 6 of those who participated in the international high-altitude expedition to Chile in 1935. Ages of the 6 ranged from 58 to 71. Two also had taken part in a high-altitude study in 1929. In their earlier years these subjects had had an increase in hemoglobin concentration beginning with their arrival at high altitude. This response has been well established, especially by the Pike's Peak party led by Haldane and the work of Hurtado and associates in Peru. On the other hand, 5 of the 6 in the 1962 party exhibited a decrease in hemoglobin concentration during the first few days. The greatest decrease was observed in the oldest subject. His hemoglobin was 88% of his sea level value after 9 days at altitude and remained below his sea level value for another week. No observations were made on blood volume; hence, we can only speculate regarding possible related changes.**

THIS REPORT deals with the concentration of hemoglobin in the blood during the early phase of acclimatization to high altitudes.

The discovery that the concentration of hemoglobin in the blood increases at high altitude is credited to Paul Bert (*1*). His observations on animals from Bolivia were supported by Viault's observations in 1890—8 years later—on man as well as animals (*2, 3*). A review of these and other early studies (not all in agreement) by Schneider (*4*) covered the literature until shortly before the Barcroft Peruvian expedition. The Pike's Peak party, led by Haldane (*5*), gave clear-cut evidence of the early increase in hemoglobin concentration in the 4 male members of the party—Haldane, Y. Henderson, Schneider, and Douglas, whose ages ranged from 29 to 51. Their daily observations

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using the Gowers-Haldane hemoglobinometer provided the first opportunity to examine the relation between age and the hematopoietic response to altitude.

No observations pertinent to our topic were made by the Barcroft study at Cerro de Pasco in Peru (*6*), except for the observation that the increase in red cell count over the sea level standard was twice as great in the Indian miners at Cerro than in either resident engineers or in the scientists 2 weeks after arriving.*

## Present Study

### Methods

During the summer of 1962 the responses of 6 subjects—Keys, Forbes, Hall, McFarland, Talbott, and Dill—at high altitudes were observed and compared with observations made on some of them at high altitudes in 1929 and 1935. Details regarding the party, the locale—White Mountain Research Station—and respiratory responses have been reported (*8, 9*). In nearly all cases hemoglobin was determined by two methods. In the first, heparinized blood was equilibrated at 37° at a $pO_2$ of 172 mm. Hg and analyzed on the Van Slyke manometric apparatus. After correction for dissolved oxygen the concentration of hemoglobin was calculated on the assumption of a combining capacity of 1.34 ml. $O_2$ per gram of hemoglobin. In the second method hemoglobin was determined colorimetrically on the Evelyn apparatus by the cyanmethemoglobin method as described by Drabkin (*10*). As a convenient short-cut the blood sample used was that contained in the Van Slyke pipet below the lower graduation. This part of each pipet used was calibrated by weighing the mercury contained in it. Thus, after introducing blood into the Van Slyke apparatus the remainder was transferred to the colorimeter tube, the lower stem of the pipet being washed several times with the Drabkin reagent. In a few instances the hematocrit on the heparinized blood was observed.

In the case of the members of the Pike's Peak expedition, moving

*In this brief historical introduction it is fitting to recognize the contribution made in this field by Van Slyke and his numerous celebrated associates. First, the Van Slyke blood gas apparatus has been an invaluable tool in modern high-altitude studies. We first employed it in Colorado in 1929, later in Chile in 1935, and at White Mountain during the summer of 1962. It was our privilege in the Harvard Fatigue Laboratory to demonstrate to Hurtado, Aste-Salazar, and associates, from Peru, and to Chiodi from Argentina, the applicability of the Van Slyke blood gas apparatus as a prime tool in high-altitude laboratories such as they subsequently established. The two-volume work by Peters and Van Slyke (*7*) is as valuable as a handbook of basic methods as it is as a source of authoritative interpretations.

averages have been employed in plotting their increments of hemoglobin concentrations; i.e., the Day 1 value is the average for Days 0, 1, and 2, and the Day 2 value, the average of Days 1, 2, and 3, etc. The results, plotted in Fig. 1, suggest both that the rate of increase in

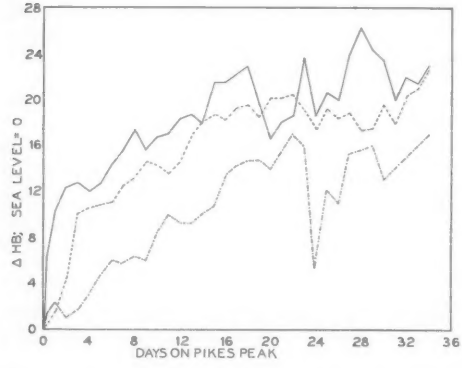


**Fig. 1.** Increase in blood hemoglobin concentrations during 34 days on Pike's Peak, at altitude of 4295 m. (*5*). Upper curve is that for Douglas, age 29; middle curve, the average response of Henderson, age 38, and Schneider, age 37; and bottom curve, response of Haldane, age 51. Curves are based on published data (*5*) using moving averages. (See text.)

hemoglobin decreases with age and also that the equilibrium value may be higher in youth than in middle age. The values for Henderson and Schneider were averaged, since they were about the same age—38 and 37, respectively. Aside from a 3-day period in the third week, their average response was below that of Douglas, aged 29. The response of Haldane, aged 51, in turn was below that of Henderson and Schneider. While they noted that the rise in hemoglobin and in the number of red corpuscles, "was especially rapid in the first two or three days in all of us except Haldane in whose case it was gradual," they did not consider age as a factor.

## Results

Table 1 lists the results of 9 experiments in 1962 in which hemoglobin was determined by the two methods described, and in which the hematocrit was observed. These results give an indication of the errors in our determinations of hemoglobin and also provide evidence that the oxygen-combining capacity of red cells is a characteristic that changes little if any with age and with the process of acclimatization to high altitudes. The mean value, approximately 20 mM/L. of red cells, is about the same as that found both at sea level and at high altitudes in 1935 (*11–13*).

**Table 1.** Hemoglobin Concentration in Blood and in Red Cells Obtained in 1962 (Present) Study

| Subject | Altitude (m.) | Hct. (%) | Hemoglobin (mM/L.) | | | |
|---|---|---|---|---|---|---|
| | | | In blood | | In red cells | |
| | | | Van Slyke | Drabkin | Van Slyke | Drabkin |
| Dill | 3800 | 40 | 7.79 | 7.53 | 19.5 | 18.8 |
| Dill | 4343 | 45.5 | 8.56 | 8.43 | 18.8 | 18.5 |
| Hall | 3800 | 49.5 | 10.09 | 9.84 | 20.4 | 19.9 |
| Keys | 3800 | 40.3 | 7.99 | 7.60 | 19.8 | 18.9 |
| Keys | 3800 | 40 | 8.06 | 8.08 | 20.2 | 20.2 |
| Keys | 4343 | 41.5 | 8.32 | 8.25 | 20.0 | 19.9 |
| Talbott | 3093 | 46 | 9.31 | 9.15 | 20.2 | 19.9 |
| Talbott | 3800 | 41.5 | 8.38 | 8.38 | 20.2 | 20.2 |
| Talbott | 4343 | 44.4 | 8.64 | 8.38 | 19.5 | 18.9 |

All the observations on hemoglobin in 1962 on Dill and Forbes are shown in Fig. 2 together with the time-course of their stays at altitude. Both showed an initial decline in hemoglobin concentration; the minimum value for Forbes was observed on the fifth day, and for Dill, on the ninth day. Fig. 3A has the corresponding records for Hall and McFarland, and Fig. 3B for Keys and Talbott. After initial slight increases in McFarland, Hall and Talbott at the Crooked Creek laboratory, hemoglobin concentrations decreased, reaching the lowest values on the fifth, third, and fourth days, respectively. In Keys there was a slight upward trend from the beginning.

Comparable observations were made on 3 of us—Dill, McFarland and Talbott—in 1935 (*11*) and also on Dill and Talbott in 1929 (*14*). The findings in 1929 and those in 1935 at the first three stations are shown in Fig. 4 together with records of the time-course of stays at altitude. There is excellent agreement between the responses of Dill

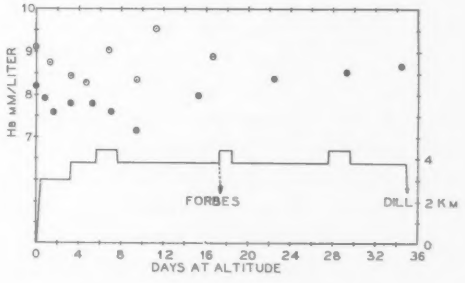


**Fig. 2.** Blood hemoglobin concentration during acclimatization to high altitude in summer of 1962. Solid circles indicate values for Dill; open circles, Forbes.

and Talbott in 1935 and their responses in 1929. Each started about 1 mM apart and showed a similar upward trend, with the exception of the 6-day value for Dill in 1929. Comparison of findings on the 3 subjects in 1935 with those of 1962 is even more clear-cut. All show a drop

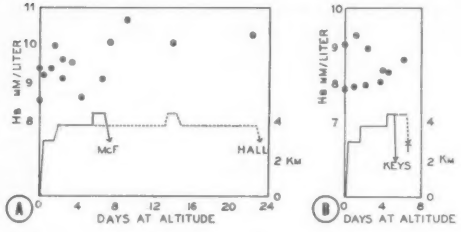


**Fig. 3.** Blood hemoglobin concentration during acclimatization to high altitude, summer of 1962. **A.** Solid circles, indicate values for MacFarland; open circles, Hall. **B.** Solid circles indicate values for Keys; open circles, Talbott.

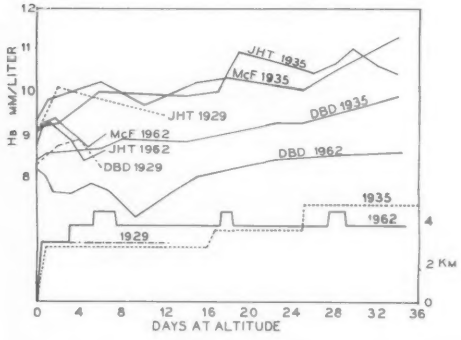


**Fig. 4.** Blood hemoglobin concentration during acclimatization to high altitudes. DBD indicates Dill; JHT, Talbott; McF, McFarland. Year of study is shown for each.

in 1962 and none a drop in 1935. There was a roughly parallel upward trend in the 3 subjects in 1935. An additional point is worth making: Dill has a relatively low hemoglobin concentration. This persists at altitude and it has persisted over a 33-year period at sea level.

## Discussion

There have not been many comparable observations in the literature, even in young men, since those of Douglas, Haldane, Henderson, and Schneider (*5*) illustrated in Fig. 1. One series has been reported by Hurtado *et al.* (*15*), and he has kindly furnished additional unpublished observations. All his subjects, Peruvian medical students, went directly to Morococha, which has an elevation of 4540 m. His results for each individual have been calculated to the sea level base line of

100 and the same has been done for our observations in the summer of 1962. The mean hemoglobin concentrations at the various times of exposure are plotted in Fig. 4. In a few cases scattered observations have been consolidated; e.g., Hurtado had a measurement on one indi-

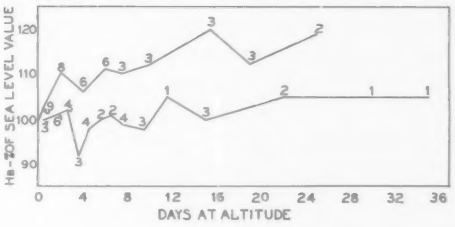


**Fig. 5.** Blood hemoglobin concentration during acclimatization. The upper curve represents mean observations reported by Hurtado *et al.* (*15*) on Peruvian medical students who went from sea level to 4540 m. Lower curve represents mean values on 6 members of 1962 study at altitudes from 3090 to 4343 m. Number of subjects is indicated for each point.

vidual at 18 days, another at 20, and a third at 22 days; the three have been averaged and the mean appears in the figure at 20 days (Fig. 5).

The results demonstrate a marked difference in the response of hemoglobin concentration to oxygen lack between young men and men ranging in age from 58 to 71. Young men from the day of arrival at altitude generally show an increase in hemoglobin. This was observed by the Pike's Peak party, in members of our group in 1929 and 1935, and in the Hurtado's medical students. On the other hand, 5 of 6 members of our group in 1962 exhibited a decreased concentration during the first days at altitude. The sixth showed a small upward trend through the first 5 days. Of the 3 subjects who spent 2 weeks or more at altitude, hemoglobin concentration eventually rose above the sea level values at 23 days (Dill), at 8 days (Hall), and at 11 days (Forbes).

We do not have observations on blood volume and hence can only speculate on the nature of related responses to high altitude. One possible interpretation of our findings is that, in the early stages of adaptation in our age range, plasma volume increases faster than red cell volume. It does not seem likely that a high rate of red cell destruction was involved, since we did not engage in much exercise during the first week. Neither is it likely that there was an upset in water balance since body weight changed little from day to day, ranging from a gain of 2.5 kg. by Talbott to a loss of 2.2 kg. by Forbes.

The novelty of our findings points to the need for further study. Frequent measurement of red cell and plasma volumes should be

coupled with daily observations of hemoglobin concentrations. Cardiac output measurements might be useful in interpreting observations on work capacity and other criteria of acclimatization. The daily urinary erythropoietin might prove quite significant. The method described by Hodgson *et al.* (*16*) has been applied by Scaro (*17*) on newcomers at 3990 m. and residents at 1260, 3990, and 4515 m. The content of erythropoietin was somewhat higher in residents at high altitude than in residents at low altitudes. It was several times higher in recent arrivals at high altitude than in residents. This approach coupled with the others mentioned above might lead to a better understanding of the relation of age to adaptation to high altitudes.

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# The Renal Clearance of Sodium Pentaborate in Mice and Men

*Lee E. Farr and T. Konikowski*

**The renal blood clearance of sodium pentaborate was observed in 8 patients undergoing neutron capture therapy for intracranial malignant tumors and was found to average 39.1 ml./min. per 1.73 sq. m. surface area.**

**The renal blood clearance was calculated for mice receiving sodium pentaborate intravenously in dosages of 25 μg./gm. body weight and 100 μg./gm., calculated as boron. The average clearance for the mice receiving the larger dose was 0.207 ml./min. for a 21-gm. mouse. The average clearance for the mice receiving the smaller dose was 0.192 ml./min. per 21-gm. mouse.**

**For the mouse, clearance calculated for a surface area of 1.73 sq. m., as was used in man, averages 40 ml./min.**

**No toxic effects on renal function were observed in mice at the higher dosage.**

IN THE COURSE of a series of experimental explorations on the effectiveness of the interaction of thermal neutrons and a target atom, Boron-10, to control malignant tumors (*1–3*), it was necessary to learn much more about the pharmacology, physiology, and toxicology of the inorganic boron salts used, namely borax, or sodium tetraborate ($Na_2B_4O_7 \cdot 10H_2O$), and sodium pentaborate ($Na_2B_{10}O_{16} \cdot 10H_2O$ (*4–6*). Since, to attain the maximum effectiveness of the treatment, a full knowledge of the distribution and excretion of the intravenously injected boron salts was necessary, renal excretion studies, among others, were carried out. From these data on excretion of boron and a knowledge of the blood boron concentration at given times after injection, it was possible to calculate a renal boron clearance, thereby permitting more effective comparison of effects on the kidney blood flow of various doses of the salt used. The experimental work dealt with

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a transplantable sarcoma in mice and intracranial malignancies in man, and, therefore, those two species were examined in relation to their boron clearances. Since the basic study deals with capture of thermal neutrons by the Boron-10 atom, all calculations here have been in terms of boron. The sodium pentaborate and sodium tetraborate salts which have been used presumably are excreted as borates. The calculation of the data as borates will not change the relationships nor the final units here reported. So, in agreement with our previously established custom we have reported in this paper the concentration of the boron compounds used in equivalent terms as boron, thereby facilitating comparisons with different boron compounds in this specific nuclear medicine context.

The mouse work was done with an inbred strain of white Swiss mice raised in the department where the work was carried out. The transplantable tumor was obtained initially by injecting methylcholanthrene intracranially into each of a group of mice, and, after about one year, selecting one of the induced neoplasms for transplantation. This neoplasm has been carried as a transplantable tumor since 1951. The patients here reported were patients who were observed as they underwent treatment by neutron capture therapy for their intracranial neoplasms. This necessitated certain limitations in the observations here reported. Other aspects of the patients' treatments will be reported elsewhere at a later date.

## Procedures

Urine collections were made on patients with an indwelling catheter required for reasons other than the renal excretion test here reported. Blood concentration was estimated by interpolation between points on a curve of analytical data, assuming a straight-line relationship between any two points in sequential time series. The blood analyses were serially performed on each patient reported. Long periods of urine collection were necessary for reasons other than those dealt with in the present report. Some patients, fairly toxic from the boron injection, experienced nausea, but vomiting was rare. The clearance calculations were carried out as $\frac{UV}{B}$ and $\frac{U\sqrt{V}}{B}$ to determine if a better fit might follow the square-root maneuver, as Van Slyke had shown was true with urea (*7, 8*).

Quite a different procedure was followed in the mice. Selected to conform to those used throughout the general and extensive explorations referred to above, they ranged in age from 5 to 6 weeks and in

body weight, from 20.5 to 21.5 gm. Urine collections in the usual sense were not possible. By use of procedure for urine collection devised by one of us (TK), each mouse was first induced to urinate by the following hand maneuver. Before injection of the boron solution, the mouse was caught deftly by pinning the tail between the fifth finger and the hypothenar eminence. The ears were then rapidly fixed between the thumb and index finger, and firm continuing pressure was put on the back by the dorsum of the middle finger; held in this position the mouse will immediately empty his bladder. This was verified by autopsy examination in a sufficient number of animals. Injection of the sodium pentaborate via the tail vein was then carried out. It is important that the needle be securely fitted into the vein and that there be no infiltration. A 0.5-ml. serological syringe with a 27-gauge, ½-inch needle was used. The volume injected in every instance was 0.01 ml./gm. observed body weight, and in this group averaged 0.21 ml. per mouse. The solution in each instance was a glucose-sodium pentaborate salt solution in water with a molar ratio of boron to glucose of 2:1. In other studies this molar ratio had been found to produce a maximum reduction in toxicity of sodium pentaborate (*6*). In our first efforts in this study each mouse in the group received a solution in which the boron concentration was 100 μg./0.01 ml. In the other group of mice, the boron concentration of the solution injected was 25 μg./0.01 ml. After injection, the animal was placed in a beaker of suitable size, usually 250 ml., the bottom of which was covered with absorbent filter paper to catch any voided urine. Not more than 45 min. prior to termination of the experiment the mouse was adroitly caught in the beaker, induced to urinate on the filter paper, and gently removed from the beaker, when the penis was ligated with a nylon surgical suture. This is a difficult maneuver, but with practice can be carried out with dexterity. The time restriction for duration of the penis ligation was established since it had been found in exploratory studies that after about 70 min. of ligation of the penis, the bladder, while remaining distended, showed a diminution rather than an increase in boron content with time. By ligating the penis within less than 1 hr. of termination, this effect was eliminated. At the appropriate time the animal was electrocuted, using a plastic box containing a sheet-nickel floor as one electrode and a series of needles protruding from the top as the other electrode. The voltage used was 120, and death was instantaneous. Immediately after death, the bladder was carefully dissected out intact and placed in a flask to which was added a known quantity of water. The bladder was then

punctured while submerged and carefully rinsed to obtain all the boron salts therein. To these washings were added combined washings of the urine-soaked filter paper which had been carefully rinsed 4–5 times with water to elute all of the boron salts. A suitable aliquot of the combined paper and bladder washings was taken for analysis. Four to five mice were sacrificed at 10-min. intervals, manipulated as above, and the results tabulated for excretory information. Both blood and urine analyses were done by our yet unpublished modification of the colorimetric method of MacDougall and Biggs (*9*). The blood concentration for the mice in each period for which a clearance was calculated was estimated from a composite graph of blood concentration for mice. Under conditions of this study, the range expected for any individual mouse was found to lie within approximately 5% of the mean. Several hundred mice had been utilized to obtain the blood curves, which were used by us in other studies for comparison to simultaneous boron analyses of different tissues. To our surpise and pleasure, we have found the mouse to be a highly predictable organism in relation to time-concentration curves for blood and tissue boron content following intravenous injections (Fig. 1).

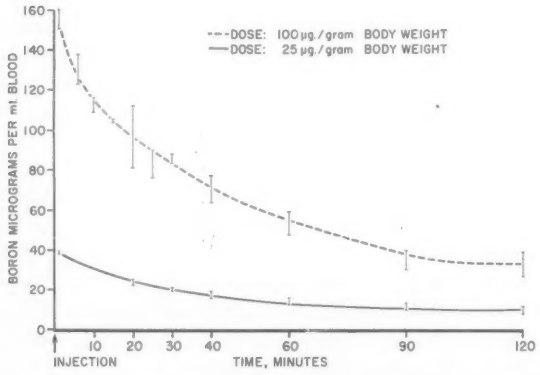


**Fig. 1.** Blood boron concentration in mouse after single I.V. injection of sodium pentaborate ($B^{10}$) glucose.

## Calculations

The boron clearances for the patients were calculated according to the urea clearance procedures of Möller *et al.* (*7*) as cubic centimeters of blood cleared of boron per 1.73 sq. m. of surface area per minute. A calculation is also shown for the same data using the square-root formula of these authors. However, in our studies no weight can be at-

tached at this time to the square-root maneuver since the augmentation limit for boron, if it exists, has not been established. Our own data suggest that for the boron compounds used this augmentation limit for boron may lie closer to 3 ml./min. than the 2 ml./min. which Van Slyke *et al.* found to hold for urea. For the mice, the data were calculated by using observed boron excretion values in micrograms per minute and dividing this figure by the estimated blood concentration at the midpoint of the excretion period. Their blood estimation was read off the blood boron time concentration curve as shown in Fig. 1. To compare the data from the mice and from the human subjects, the data for the mice were calculated on the basis of surface area using Meeh's formula: $SA = 0.114\ \sqrt[3]{W^2}$, where $SA$ is the surface area in square meters and $W$ is the observed weight of the animal in kilograms. The number 0.114 is the characteristic constant for mice as calculated by Rubner (*10*). This resulting figure was then adjusted to a surface area of 1.73 sq. m.

## Results

Data from the mice are shown in Tables 1 and 2 and Fig. 1; data from the patients are shown in Table 3. When the salt injection was sodium pentaborate and the boron dose was 35 mg./kg. body weight,

**Table 1.** URINARY BORON EXCRETION IN MICE

| | *Boron excreted per mouse (μg.)* | | | | | | | |
|---|---|---|---|---|---|---|---|---|
| | *0-10 min.* | *0-20 min.* | *0-30 min.* | *0-40 min.* | *0-50 min.* | *0-60 min.* | *0-70 min.* | *0-90 min.* |
| BORON DOSE:* 25 μG./GM. BODY WEIGHT | | | | | | | | |
| | 57.0 | 108.0 | 114.8 | 179.5 | 229.5 | 251.5 | 244.0 | 286.0 |
| | 54.8 | 100.8 | 111.0 | 178.0 | 203.5 | 265.0 | 297.5 | 323.0 |
| | 47.5 | 101.5 | 115.8 | 181.8 | 248.5 | 225.0 | 216.0 | 315.0 |
| | | 94.5 | 139.3 | 177.5 | 176.5 | 231.5 | 208.0 | |
| | | | 135.5 | 171.3 | 189.5 | 261.5 | | |
| Av. | 53.1 | 101.2 | 123.7 | 177.6 | 209.5 | 246.9 | 251.1 | 301.7 |
| BORON DOSE:* 100 μG./GM. BODY WEIGHT | | | | | | | | |
| | 197.0 | 480.0 | 527.0 | 854.0 | 870.0 | — | — | — |
| | 192.0 | 446.0 | 558.0 | 835.0 | 976.0 | — | — | — |
| | 197.0 | 441.0 | 560.0 | 840.0 | 882.0 | — | — | — |
| | 176.0 | 397.0 | 528.0 | 752.0 | 952.0 | — | — | — |
| | 226.0 | 322.0 | 460.0 | 654.0 | — | — | — | — |
| Av. | 197.6 | 417.2 | 526.5 | 787.0 | 920.0 | — | — | — |

*Given intravenously at time zero.

**Table 2.** Boron Clearance Calculation in Mice

| Collection period (min. post injection) | Interval Boron excretion | | B Blood | UV/B Clearance |
|---|---|---|---|---|
| | γ/period | UV γ/min. | γ/ml. | ml./min. |
| **Boron Dose: 25 γ/gm. Body Weight** | | | | |
| 10-20 | 48.1 | 4.81 | 26.2 | 0.184 |
| 20-30 | 22.5 | 2.25 | 22.5 | 0.100 |
| 30-40 | 53.9 | 5.39 | 19.0 | 0.284 |
| 40-50 | 31.9 | 3.19 | 16.5 | 0.193 |
| 50-60 | 37.4 | 3.74 | 14.5 | 0.258 |
| | | | | Av. 0.204 |
| 10-30 | 70.6 | 3.53 | 24.0 | 0.147 |
| 20-40 | 76.4 | 3.82 | 20.7 | 0.185 |
| 30-50 | 85.8 | 4.29 | 17.2 | 0.249 |
| 40-60 | 69.3 | 3.47 | 15.5 | 0.224 |
| 50-70 | 41.6 | 2.08 | 14.0 | 0.149 |
| | | | | Av. 0.191 |
| 10-40 | 124.5 | 4.15 | 22.5 | 0.184 |
| 20-50 | 108.3 | 3.61 | 19.0 | 0.190 |
| 30-60 | 123.2 | 4.11 | 16.5 | 0.249 |
| 40-70 | 73.5 | 2.45 | 14.5 | 0.169 |
| 60-90 | 54.8 | 1.63 | 12.5 | 0.130 |
| | | | | Av. 0.184 |
| **Boron Dose: 100 γ/gm. Body Weight** | | | | |
| 10-20 | 219.6 | 21.96 | 104 | 0.211 |
| 20-30 | 109.3 | 10.93 | 90 | 0.121 |
| 30-40 | 160.5 | 16.05 | 76 | 0.211 |
| 40-50 | 133.0 | 13.30 | 65 | 0.205 |
| | | | | Av. 0.187 |
| 10-30 | 328.9 | 16.5 | 98 | 0.168 |
| 20-40 | 369.8 | 18.5 | 83 | 0.223 |
| 30-50 | 393.5 | 18.4 | 68 | 0.271 |
| | | | | Av. 0.221 |
| 10-40 | 589.4 | 19.65 | 90 | 0.218 |
| 20-50 | 502.8 | 16.76 | 76 | 0.221 |
| | | | | Av. 0.219 |
| 10-50 | 732.4 | 18.31 | 83 | 0.221 |

| | | | | |
|---|---|---|---|---|
| 10-50 | 156.4 | 3.91 | 20.7 | 0.189 |
| 20-60 | 145.7 | 3.64 | 17.2 | 0.212 |
| 30-70 | 127.4 | 3.19 | 15.5 | 0.206 |
| | | | | Av. 0.202 |
| 10-60 | 193.3 | 2.88 | 19.0 | 0.152 |
| 20-70 | 149.9 | 2.99 | 16.5 | 0.181 |
| 40-90 | 124.1 | 2.80 | 13.5 | 0.207 |
| | | | | Av. 0.180 |
| 10-70 | 198.0 | 3.30 | 17.2 | 0.192 |
| 30-90 | 178.0 | 2.83 | 14.0 | 0.202 |
| | | | | Av. 0.197 |
| 20-90 | 200.5 | 2.86 | 14.5 | 0.197 |
| 10-90 | 248.0 | 2.76 | 15.5 | 0.178 |
| | | | | Av. 0.187 |
| | | | General Average | 0.192 |

General Average 0.207

**Table 3.** Calculated Renal Blood Boron Clearances in Patients

| Patient | | | | Time elapsed (min.) | Urine vol. (ml.) | V Urine vol./min. (ml./min.) | Vc Corrected urine vol./min. (ml./min.) | Boron conc. (mg./ml.) | | Clearance (ml./min.) | | |
|---|---|---|---|---|---|---|---|---|---|---|---|---|
| No. | Sex | Age | ft | | | | | U In urine | B In blood | U B | UVc B | UVVc B |
| 80240 | M | 43 | .895 | 240 | 220 | 0.917 | 0.821 | 0.956 | 0.055 | 17.4 | 14.3 | 15.8 |
| | | | | 240 | 345 | 1.438 | 1.287 | 0.712 | 0.050 | 14.2 | 18.3 | 16.2 |
| | | | | 794 | 794 | 0.793 | 0.709 | 1.084 | 0.039 | 27.8 | 19.7 | 23.4 |
| | | | | 555 | 555 | 0.8 | 0.790 | 1.220 | 0.023 | 53.0 | 41.9 | 47.2 |
| | | | | | | | | | | | Av. 23.6 | |
| 80178 | M | 37 | .87 | 240 | 208 | 0.87 | 0.77 | 2.75 | 0.042 | 65.5 | 50.4 | 57.6 |
| | | | | 240 | 100 | 0.42 | 0.37 | 2.49 | 0.034 | 73.2 | 27.1 | 44.5 |
| | | | | 240 | 260 | 1.08 | 0.94 | 1.47 | 0.021 | 70.0 | 65.8 | 67.9 |
| | | | | 720 | 1737 | 2.41 | 2.10 | 0.31 | 0.014 | 22.1 | 46.4 | |
| | | | | | | | | | | | Av. 47.4 | |
| 80177 | M | 66 | 1.03 | 240 | 140 | 0.58 | 0.60 | 1.47 | 0.048 | 30.6 | 18.4 | 23.8 |
| | | | | 240 | 128 | 0.53 | 0.55 | 1.54 | 0.039 | 39.5 | 21.7 | 29.3 |
| | | | | 240 | 863 | 3.60 | 3.71 | 0.34 | 0.028 | 12.1 | 44.9 | |
| | | | | 720 | 912 | 1.27 | 1.31 | 0.58 | 0.015 | 38.7 | 50.7 | 44.3 |
| | | | | | | | | | | | Av. 33.9 | |

| | | | | | | | | | | | | |
|---|---|---|---|---|---|---|---|---|---|---|---|---|
| 80194 | M | 32 | .94 | 240 | 166 | 0.69 | 0.65 | 1.82 | 0.051 | 35.7 | 23.2 | 28.8 |
| | | | | 240 | 160 | 0.67 | 0.63 | 1.05 | 0.039 | 26.9 | 16.9 | 21.4 |
| | | | | 240 | 378 | 1.58 | 1.49 | 1.19 | 0.030 | 29.7 | 59.2 | 48.4 |
| | | | | | | | | | | | Av. 33.1 | |
| 80162 | M | 52 | .86 | 240 | 223 | 0.93 | 0.80 | 2.60 | 0.033 | 78.8 | 63.0 | 70.5 |
| | | | | 240 | 220 | 0.92 | 0.79 | 1.68 | 0.025 | 67.2 | 53.1 | 59.8 |
| | | | | 720 | 185 | 0.26 | 0.22 | 1.50 | 0.014 | 107.1 | 23.6 | 50.4 |
| | | | | | | | | | | | Av. 46.6 | |
| 80175 | M | 67 | .99 | 230 | 730 | 3.17 | 3.14 | 0.774 | 0.033 | 23.5 | 73.8 | |
| | | | | 250 | 665 | 2.66 | 2.63 | 0.412 | 0.028 | 14.7 | 38.7 | |
| | | | | 240 | 407 | 1.70 | 1.68 | 0.466 | 0.023 | 20.3 | 34.1 | 26.3 |
| | | | | 720 | 825 | 1.15 | 1.14 | 0.490 | 0.014 | 35.0 | 39 9 | 37.4 |
| | | | | | | | | | | | Av. 46.6 | |
| 80158 | F | 45 | 1.27 | 1140 | 2820 | 2.47 | 3.14 | 0.216 | 0.012 | 18.0 | 56.5 | |
| 80154 | M | 57 | — | 420 | 341.0 | 0.812 | 0.812 | 1.61 | 0.035 | 46.0 | 37.4 | 41.5 |
| | | | | 650 | 419.0 | 0.645 | 0.645 | 1.04 | 0.018 | 57.8 | 37.3 | 46.5 |
| | | | | | | | | | | | Av. 37.3 | |
| | | | | | | | | | | | General Average: 39.1 | |

*Each patient received at time zero an intravenous injection of sodium pentaborate in glucose in a volume of 100 ml., providing a boron dose of 35 mg./kg. body weight. Clearances were observed during and after neutron capture therapy for intracranial malignant neoplasms.

†Ratio: calculated surface area of patient to 1.73.

the renal clearance for boron was, from these data, found to average 39.1 ml./min. for a man with a surface area of 1.73 sq. m. The variations were not greater than one would expect from the clinical conditions of the patients concerned. It is not known whether comparable variations in urea or other clearances were occurring simultaneously.

The data for the mice are in surprising agreement when one takes into account the manner of estimation. The average clearance for a 21-gm. mouse was 0.207 ml./min. when the boron administered was 100 mg./kg. and 0.192 ml./min. when the boron dose was 25 mg./kg. Calculated per square meter of surface area, this clearance became 23 ml./sq. m./min. This is over twice the urea clearance one of us observed in rats (*11*). When this clearance of 23 ml./sq. m./min. is adjusted for 1.73 sq. m. the clearance became 40.0 ml./min. This is in astonishing agreement with our patient clearance of 39.1 ml./min. With such close agreement seen in the clearance in mice after the two doses used, it is evident that there is no apparent renal toxic effect from administration of a boron dose (100 mg./kg.) that would be fraught with grave toxic consequences in man.

Comparison of the clearances observed in the mouse and in man suggest that the 35 mg./kg. dose of boron in man is not obviously toxic. From general clinical observations we believe that a dose of this size is quite safe, and only a minimum of toxic manifestation will ensue. It is unfortunate that we were unable to determine simultaneous urea clearances in the patients reported in this study, so that the validity of one formula over another or similarity in the augmentation limit could have been established.

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# High-Temperature Operation of Oxygen Electrode Giving Fast Response for Respiratory Gas Sampling

*John W. Severinghaus*

**The 98% response time of the membrane-covered Clark-type polarographic oxygen electrode may be reduced to 0.4 sec. by heating the electrode and the gas sample flowing past it to 80° when the electrode is covered with 0.25-mil (6 $\mu$) Teflon membrane. The design of the microcathode electrode has been modified slightly to prevent drying out of the electrolyte and pressure changes within the electrolyte cavity. The relationship of output current to temperature approaches a plateau at this temperature which suggests that for reasonably accurate operation, temperature regulation may not be as crucial as it is at body temperature.**

THE RATE of response of the membrane-covered Clark-type* polarographic oxygen electrode is limited by the rate of diffusion of oxygen through the membrane. Kreuzer (*1*) and Staub (*2*) have used Teflon membranes 5 $\mu$ thick to reduce response times to about 1 sec. Kreuzer, using his catheter electrode in the airway, was able to record approximate oxygen plateaus in expired alveolar gas during slow respiration and reported 95% response in 1 sec. However, the electrode output current was sensitive to airway temperature which varied from inspiration to expiration. This variation is in part due to the high-temperature permeability coefficient of Teflon (*3*). At higher temperatures the current derived at a given oxygen tension increases and the response time decreases. It has therefore been found possible to shorten the response time and stabilize the temperature by placing the electrode in a thermostated metal cuvet (kept at elevated temperature) through which a gas sample is pulled.

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## Methods

The commercially available Clark-type $pO_2$ electrodes with the micro platinum cathodes (25-$\mu$ diameter) have almost no electrolyte cavity and no opening for relief of pressure from this electrolyte space. When temperature is elevated, they tend to dry quickly, and dissolved air in the electrolyte escapes to form gas bubbles which increase the pressure within the electrolyte space. An electrode was constructed with dimensions comparable to the standard electrodes but with an electrolyte chamber having openings for filling and for pressure relief (Fig. 1). The platinum cathode diameter was 25 $\mu$. For high-temperature operation it was found that if the glass surrounding the platinum were too smooth, evaporation of water through the Teflon membrane resulted in loss of continuous film between the anode and cathode. If the

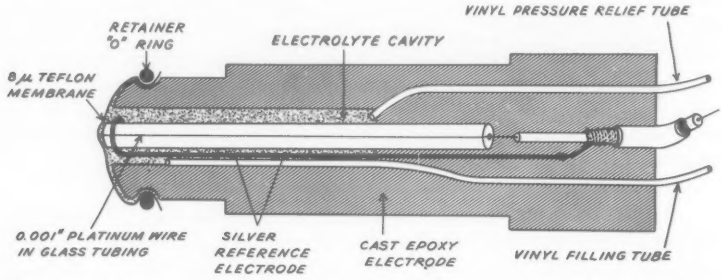


**Fig. 1.** Modification of oxygen electrode for use at high temperature. Catheters built into electrode permit it to be filled or flushed with electrolyte and prevent pressure build-up due to heating and bubble formation.

surface was too rough, edgewise diffusion of oxygen resulted in a slow component or tail in the response. The surface was prepared by very gentle grinding under water on a fine-grain carborundum stone. Teflon membranes of 6–8 $\mu$ were used. A buffered electrolyte, prepared by adding 1 ml. of saturated KCl to 30 ml. of undiluted, pH 7 phosphate buffer stock concentrate, was used to eliminate the effect of $CO_2$ and the nonlinearity seen at high $pO_2$. A commercially available,* stainless-steel, water-jacketed, 25-$\mu$l. cuvet intended for capillary blood $pO_2$ analysis was slightly modified (Fig. 2). Temperature was regulated to 0.1° by a circulating thermostat. Gas samples were pulled through the cuvet at 100–150 ml./min. using PE 90 polyethylene catheter. The hub of the No. 19 needle inlet was cut off to permit the sampling cathe-

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ter to be mounted directly on the needle tubing. The output of the electrode was amplified and recorded using a Grass, low-level DC preamplifier* and ink writer. A suitable input coupling circuit is indicated in Fig. 3.

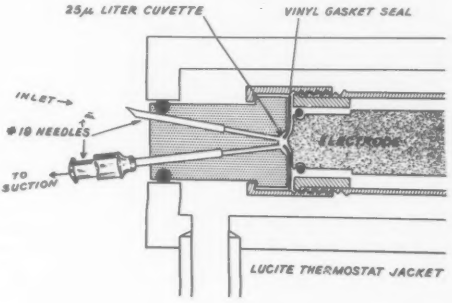



**Fig. 2.** A 25-μl. cuvet with water jacket. Electrode is sealed in by vinyl gasket between cuvet and front of electrode. Inlet and outlet were drilled out to accept needle tubing.

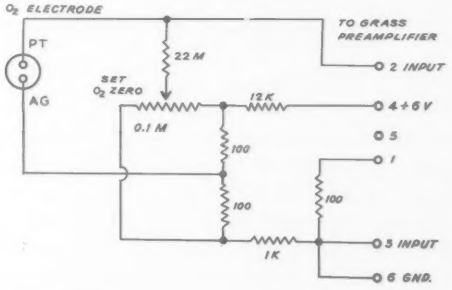



**Fig. 3.** Circuit used to provide 0.50 v for driving electrode and a zero $O_2$ adjustment and to couple electrode into low-level DC preamplifier and recorder.

## Results

The output current of the electrode with 8-μ Teflon membrane at 70° in air was approximately $3 \times 10^{-9}$ amp. The polarographic plateau at higher temperatures, as shown in Fig. 4, was shifted to lower voltage. At 80° the zero $O_2$ (background) current was found to rise rapidly with polarizing voltages above 0.5 v, whereas at 37° this occurred well above 0.8 v. With time, this background current tended to fall towards zero (circled points on Fig. 4), resulting in unstable readings. At 0.5 v, background current fell to a constant value within a few minutes of operation. Accordingly, 0.5 v was selected for the following studies.

The effect of temperature on the response time expressed both as the

*Model 5P1G.

time constant (time to 63% of maximum) and as the 98% response (four time constants if response is exponential) is shown in Fig. 5. The electrode responds about twice as fast at 70° to 80° as at 37°. The current through the electrode increased about 50% over the same range and was less sensitive to temperature change at the high than at

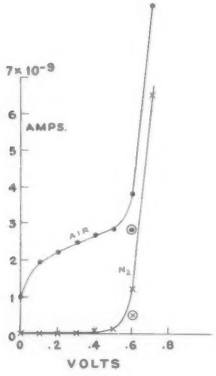



**Fig. 4.** Polarogram of electrode at 80° with air and $O_2$ free gas. Circled points at 0.6 v were obtained after waiting 30 min., indicating gradual fall in background current. This was avoided by operation at 0.5 v.

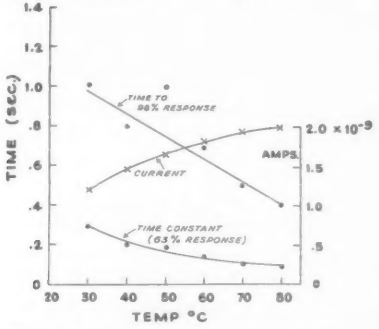
the low temperature range. This is of interest in that small temperature changes will produce less drift at high temperature. The response of the oxygen electrode at 80° to an instantaneous change from air to nitrogen and back to air is compared in Fig. 6 with the response of an infrared $CO_2$ analyzer* to a sudden introduction and withdrawal of $CO_2$. The response was found to be approximately a single exponential provided the surface of the glass surrounding the platinum was uniformly ground, eliminating pock marks and cracks at the edge of the platinum; such irregularities tended to introduce a slow component in the response curve. The electrode was linear to within 1% at 80° over the range 0-100% oxygen (Fig. 7).

The $pO_2$ is, of course, dependent on the water vapor concentration in the gas sample. In air saturated at body temperature, water vapor decreased the current about 5% (Fig. 8). When using polyethylene sampling catheters, there was a considerable delay in response to water vapor changes particularly in substituting dry for wet gas. Water vapor clung to the inside of the tubing and cuvet and continued to saturate the inflowing air for many seconds after the shift to dry gas. For practical purposes, when sampling an air stream through a catheter, it

*Model LB-1, Beckman Instruments, Inc., Spinco Division.

may be assumed that the water vapor pressure does not change between inspiratory and expiratory readings because of this lag effect.

At high operating temperatures it was more difficult to obtain stable electrode operation. Electrodes have been assembled which have remained stable over many hours and in which no membrane change has



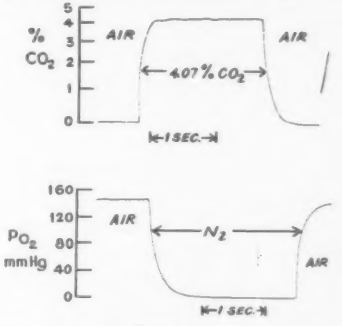




**Fig. 5** (*left*). Effect of temperature upon response time and current of modified $pO_2$ electrode. Teflon membrane, 8$\mu$; sampling catheter length, 5 cm.; gas flow, 150 ml./min. **Fig. 6** (*right*). Comparison of response to step change in concentration of oxygen electrode at 80° with infrared $CO_2$ analyzer, using low-pressure, high-velocity sampling with a 5-cm. catheter.

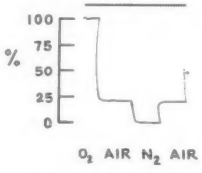
been necessary from day to day. However, with the extremely thin membranes and high temperatures the membranes may develop holes and fail within a few minutes after assembly. Some of the sources of failure have been identified as follows.

1. Gas bubbles arising in the electrolyte from dissolved gases break the continuity of the electrical cell. To avoid this, it has been found helpful to drive off the dissolved gases by boiling the electrolyte before injecting it into the electrode.

2. Negative pressure in the cuvet ruptures the very thin membrane. Hence, negative pressure in the cuvet must be held at a minimum. If a constant flow suction is used the membrane will rupture if the sampling tip orifice is temporarily obstructed for even a fraction of a second, as it may be when the sampling needle tip is pushed through a rubber mouthpiece. Therefore, the suction to the electrode should be stopped when any manipulation of the sampling tip is being done.

3. Particulate dirt punctures the membrane. To avoid this, the cuvet and the vinyl gasket as well as the plastic and glass portions of the electrode which come in contact with the membrane must be kept scrupulously clean and smooth.

4. The membrane is stretched in applying it over the tip of the electrode. This must not be done not only because of the danger of creation of holes but because, when the membrane presses too tightly on the electrode tip, the thin film of water may dry out, resulting in a reduction of the output current to zero.



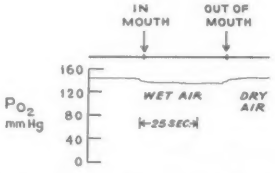




**Fig. 7** (*left*). Linearity of $O_2$ electrode (80°). **Fig. 8** (*right*). Effect of water vapor on hot $O_2$ electrode. Water vapor saturated at body temperature by sampling from closed mouth filled with air reduced reading about 5%. Time markers appear displaced because of curvilinear recording. With longer (20-cm.) sampling catheter, water vapor variation during respiratory cycle was avoided due to water vapor's clinging to catheter walls.

An upward drift of the current in the electrode generally occurs after long periods of operation. It is believed that this results from the electrolytic deposition of trace metals, probably mostly silver, on the edges of the cathode, increasing the effective area of the cathode. These trace metals may be removed by mild polishing of the tip with a kitchen abrasive or may require the use of the fine carborundum stone.

Drift of the electrode tends to be somewhat more than that observed at 37° operation principally because of the tendency of the electrolyte film to dry. It was found important to avoid change in flow rate through the cuvet since such changes altered both electrode tip temperature and total gas pressure.

The electrode may be calibrated in use if the inspired oxygen concentration is known. Since zero does not ordinarily drift, the gain of the amplifier may be reset to make the recorder deflect correctly during inspiration. In an experiment in which the inspired concentration was

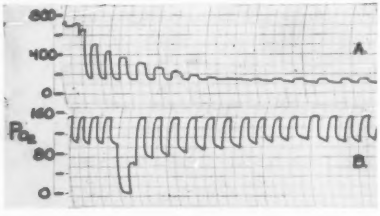



**Fig. 9.** Recording of $pO_2$. **A** indicates trace after washout with air after breathing 100% $O_2$; **B**, effect of single breath of 100% $N_2$.

varied we have used a paramagnetic recording oxygen analyzer* to record inspired concentration continuously, and have adjusted the calibration of the rapid responding polarographic electrode to agree with this reading during inspiration.

## Discussion

An oxygen pressure sensor responding sufficiently rapidly to follow oxygen tension changes in the airway makes possible recordings comparable to those now commonly available with the infrared $CO_2$ analyzer (Fig. 9). The other instrument available commercially which can offer fast response to changing oxygen tensions is the mass spectrometer, which is not available in most laboratories because of its high cost and complexity of operation. The oxygen electrode, with a suitable cuvet and circulating thermostat, is available for less than $500. The plateau of oxygen electrode current with temperature at the high-temperature range suggests the construction of a self-thermostated cuvet, which is now under construction in this laboratory.

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*Model F, Beckman Instruments, Inc.

# Competition Between Calcium, Strontium, and Magnesium for Absorption in the Isolated Rat Intestine

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**Rats 1–2 mo. old were placed on a low calcium or a low calcium and low magnesium diet. Absorption in isolated rat intestine was studied in vitro. Duodenal segments which actively took up and actively transported $Ca^{45++}$ well, actively took up and actively transported $Sr^{85++}$ well; where $Ca^{45++}$ active uptake and active transport was poor, that of $Sr^{85++}$ was low. The uptakes of $Ca^{45++}$, $Sr^{85++}$, and $Mg^{28++}$ by the gut tissue were greater than the amounts transported. $Ca^{++}$, $Sr^{++}$, and $Mg^{++}$ decreased the active uptake of $Ca^{45++}$ and $Sr^{85++}$ by the duodenum. $Ca^{++}$ decreased the rate of active transport to the serosal media of $Ca^{45++}$ and $Sr^{85++}$. $Ca^{++}$ was more effective in decreasing the active uptake of $Sr^{85++}$ than was $Sr^{++}$. The active uptake of $Ca^{45++}$ by the ileum was reduced by $Mg^{++}$ and $Sr^{++}$. The active uptake of $Mg^{28++}$ by the ileum was reduced by $Ca^{++}$. The rate at which $Mg^{28++}$ crosses and is taken up by the small intestine increases as one progresses from the proximal duodenum to the distal ileum. $Ca^{++}$ was actively transported by the colon. $Ca^{++}$, $Sr^{++}$, and $Mg^{++}$ appear to share a common pathway for absorption by the gut, but in the various areas of the intestine the rate of absorption of a particular divalent cation and the degree of competition between the divalent cations varies. Therefore, the absorption of a particular cation may involve also other pathways.**

DURING the past 53 years, it has become increasingly apparent that there exists an interrelationship between $Ca^{++}$, $Mg^{++}$ and $Sr^{++}$ for transport in both the intestine and the renal tubule. The studies of Berglund and Forster (*1*) indicated that there might be competition between $Ca^{++}$ and $Mg^{++}$ for transport by the renal tubule of an

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aglomerular teleost. Recently, Alcock and MacIntyre (*2, 3*) postulated that $Ca^{++}$ and $Mg^{++}$ compete for transport both in the intestine and in the renal tubule of rats. Therefore, the concept is suggested that divalent cations compete at different sites along the lumen and substitute for each other to varying degrees for active absorption from, and active secretion into the gut, and for active tubular reabsorption in the kidney. The present study was carried out to determine if there is a competitive relationship between $Ca^{++}$, $Mg^{++}$, and $Sr^{++}$ for active absorption by the isolated rat intestine.

## Material and Methods

Albino rats obtained from the Charles River Breeding Laboratories were used in all the experiments. In Experiment 1, male animals, aged 2.5 months and weighing 200 gm., were used; these received a low calcium diet (Nutritional Biochemicals Corp.) for 10 days prior to the experiment. In Experiment 2, female rats, aged 4 weeks and weighing 80 gm., were used after being fed a low calcium and low magnesium diet (Nutritional Biochemical Corp.) for 5 days. In all cases the nonfasted rats were sacrificed by a blow on the head. Portions of the proximal duodenum, mid-small intestine, distal ileum, or colon, each 8 cm. long, were removed from each rat. A modification of the method of Wilson and Wiseman (*4*) was used in handling the gut. The segments were rinsed in normal saline at room temperature, everted, and rinsed again; they were then blotted on a paper towel and the washing solution gently expressed from the lumen. Each segment was then weighed, tied at one end with a silk thread, and 1 ml. of the appropriate medium placed into the serosa-lined lumen. The gut sac was tied at the other end and the whole immersed in 3 ml. of medium in a test tube at 37°. The tissues were incubated at 37° for 1.5 hr., with oxygen continuously bubbled through the medium via a plastic catheter. At the conclusion of the incubation, about 0.8 ml. of the inside medium and 2.3 ml. of the outside medium were recovered. Incomplete recovery of media is attributed to adherence to the surface of the gut wall, adherence to the glassware, and evaporation.

Tris-hydroxymethyl aminomethane-HCl (Tris) (Sigma Chemical Co.) pH 7.4, 0.05M, was the buffer used in the incubating media. Tris was chosen since preliminary experiments showed that its presence did not alter the concentration of total divalent cation ($Mg^{++}$, $Ca^{++}$, or $Sr^{++}$) required for the precipitation of magnesium, calcium, or strontium phosphate at pH 7.4; from these data it was concluded that these

cations did not form a poorly dissociated compound with Tris and, hence, that it was a suitable buffer.

The incubating media were as follows.

### Experiment 1

*Serosal medium* 0.05M tris buffer, pH 7.4, 0.106 M NaCl.

*Mucosal medium, control* 0.05M tris buffer, pH 7.4, 0.106 M NaCl; $10^{-8}$M $SrCl_2$, $10^{-8}$M $CaCl_2$, $Sr^{85++}$ 0.02 $\mu$c/ml., $Ca^{45++}$ 0.1 $\mu$c/ml. (A minimum amount of $CaCl_2$ and $SrCl_2$ was added to the medium to prevent significant adsorption of $Ca^{45++}$ and $Sr^{85++}$ onto the glassware.)

*Mucosal medium, experimental* 0.05 M tris buffer, pH 7.4, 0.091 M NaCl; 0.01M $CaCl_2$ or 0.01M $SrCl_2$ or 0.01 M $MgCl_2$, $Sr^{85++}$ 0.02 $\mu$c/ml., $Ca^{45++}$ 0.1 $\mu$c/ml. (NaCl concentration was varied to maintain isotonicity.)

### Experiment 2

The media used in Experiment 2 were the same as those of Experiment 1 except that $Mg^{28++}$ and $Mg^{24++}$ were substituted for $Sr^{85++}$ and $Sr^{87++}$, and the concentrations of the divalent cations were as follows.

*Mucosal medium, control* $10^{-4}$ M $CaCl_2$, $2 \times 10^{-4}$ M $MgCl_2$.

*Mucosal medium, experimental* $1.7 \times 10^{-4}$ M $CaCl_2$, $3 \times 10^{-4}$ M $MgCl_2$, 0.011 M or 0.033 M of $SrCl_2$, $CaCl_2$, or $MgCl_2$. The intestine (*5*) contains $3.5 \times 10^{-3}$ moles calcium per kilogram and $3.5 \times 10^{-3}$ moles magnesium per kilogram.

In Experiment 1, the effect of 0.01 M $CaCl_2$, $SrCl_2$, and $MgCl_2$ on the uptake and transport of $Ca^{45++}$ and $Sr^{85++}$ by the duodenum were measured. In Experiment 2, the uptake and transport of $Ca^{45++}$ and $Mg^{28++}$ by the duodenum, jejunum, ileum, and colon were measured; also, the effect on this process of 0.033 M and 0.011 M $CaCl_2$, $SrCl_2$, and $MgCl_2$ was measured in the duodenum and ileum. Duplicate experiments were performed using the conditions of Experiment 1; 3–4 rats were used for each variable tested on each occasion. Results for Experiment 2 were obtained from a single experiment; 3–4 rats were used for each variable tested.

The analytic procedure involved pipetting 0.1 ml. of the inside medium and 0.1 ml. of the outside medium directly into separate sample holders. The time required for 10,000 counts was measured for each sample and corrections were made for background activity. Either $Ca^{45++}$ was present with $Sr^{85++}$, or $Ca^{45++}$ was present with $Mg^{28++}$.

$Sr^{85++}$ and $Mg^{28++}$ were counted in the well scintillation counter, which did not detect any significant amount of $Ca^{45++}$. The $Ca^{45++}$ was counted in a Tracerlab end-window Geiger-Muller detector, which did not measure a significant amount of $Sr^{85++}$. $Mg^{28++}$ was allowed to decay to an unmeasurable level before $Ca^{45++}$ was measured.

The entire gut segment was rinsed thoroughly with normal saline, then placed in a glass tube and counted for either $Sr^{85++}$ or $Mg^{28++}$. Then it was digested with 7 ml. of a mixture of 3 parts conc. $HNO_3$ and 1 part 60% $HClO_4$ on a hot sand bath and evaporated to dryness. The ash was then dissolved in 0.1N HCl and an aliquot pipetted directly into a planchet, dried, then counted for $Ca^{45++}$.

The results were expressed as a ratio ($^S/_M$) of counts per minute in 0.1 ml. serosal medium (inside) to counts per minute in 0.1 ml. mucosal medium (outside), and as a ratio ($G/M$) of counts per minute in 0.1 gm. fresh gut tissue to counts per minute in 0.1 ml. of mucosal medium (outside). The results were analyzed by means of Student's t-test (*6*).

Total calcium and magnesium were estimated by means of a flame spectrophotometer (*7, 8*).

## Results

Using the method previously described for arbitrary expression of the results, it was found that (Table 1) those duodenal segments which actively took up and actively transported $Ca^{45++}$ well, also actively took up and actively transported $Sr^{85++}$ well; where $Ca^{45++}$ active uptake and active transport were poor, that of $Sr^{85++}$ was low. Furthermore, the active uptake of $Ca^{45++}$ and $Sr^{85++}$ was greater than the amount which was actively transported.

$Ca^{++}$, $Sr^{++}$, and $Mg^{++}$ at 0.01 M decreased the rate of active uptake of $Ca^{45++}$ and $Sr^{85++}$ by the duodenum (Tables 2 and 3); also, 0.01 M $Ca^{++}$ decreased the rate of active transport to the serosal media of both $Ca^{45++}$ and $Sr^{85++}$. Moreover, $Ca^{++}$, $Sr^{++}$, and $Mg^{++}$ had no effect on the rate at which $Mg^{28++}$ crossed the duodenum (Table 3). $Mg^{++}$ reduced the mean uptake of $Mg^{28++}$ by the duodenum.

The rate at which $Ca^{45++}$ traverses the ileal wall was reduced by $Ca^{++}$, but it was not reduced by $Sr^{++}$ or $Mg^{++}$ (Table 4). The uptake of $Ca^{45++}$ by the ileum was significantly reduced by $Ca^{++}$, $Sr^{++}$, and $Mg^{++}$. The uptake of $Mg^{28++}$ by the ileum was significantly reduced by $Ca^{++}$ and $Mg^{++}$.

The rate at which $Mg^{28++}$ crosses the ileal and jejunal wall is significantly more rapid than the rate at which it crosses the duodenal wall

**Table 1.** EFFECT OF $Mg^{++}$, $Sr^{++}$, AND $Ca^{++}$ ON TRANSPORT AND UPTAKE OF $Sr^{85++}$ AND $Ca^{45++}$ IN DUODENAL SEGMENTS

| | *Strontium-85* | | *Calcium-45* | |
|---|---|---|---|---|
| *Rats* | *S/M** | *G/M** | *S/M** | *G/M** |
| | | CONTROLS | | |
| 1 | 1.8 | 1.8 | 6.9 | 6.6 |
| 2 | .60 | .96 | 2.0 | 3.8 |
| 3 | .64 | .95 | 1.8 | 2.6 |
| 4 | .40 | 1.3 | .62 | 2.2 |
| 5 | .53 | 1.8 | 1.6 | 4.2 |
| 6 | 1.1 | 2.1 | 3.8 | 7.4 |
| | | 0.010 M $MgCl_2$ | | |
| 7 | .36 | .50 | 2.4 | 2.5 |
| 8 | .45 | .55 | .94 | 1.4 |
| 9 | .49 | .73 | 1.7 | 2.7 |
| 10 | .44 | 1.1 | .72 | 2.0 |
| 11 | .43 | .74 | .72 | 1.3 |
| 12 | .43 | .97 | .87 | 2.2 |
| | | 0.010 M $SrCl_2$ | | |
| 13 | .57 | .61 | 1.9 | 1.8 |
| 14 | .55 | .75 | 1.7 | 1.8 |
| 15 | .53 | .68 | .96 | .92 |
| 16 | .53 | 1.1 | .94 | 2.9 |
| 17 | .48 | 1.1 | .78 | 2.7 |
| 18 | .51 | .83 | .53 | 1.8 |
| | | 0.010 M $CaCl_2$ | | |
| 19 | .38 | .55 | .47 | .63 |
| 20 | .33 | .91 | .44 | 1.1 |
| 21 | .40 | .84 | .51 | .92 |
| 22 | .27 | .59 | .34 | .77 |
| 23 | .33 | — | .39 | — |
| 24 | .29 | .59 | .30 | 1.0 |
| 25 | .20 | .87 | .33 | 1.5 |

*See text.

(Table 4). The uptake of $Mg^{28++}$ by the ileum, jejunum, and colon was greater than the uptake of $Mg^{28++}$ by the duodenum (Table 4). Simultaneous tracer studies with $Mg^{28++}$ and $Ca^{45++}$ showed that, although $Mg^{28++}$ was not actively transported or actively taken up by the duodenum, $Ca^{45++}$ was actively transported and actively taken up by the same duodenal segments.

**Table 2.** Effect* of $Mg^{++}$, $Sr^{++}$ and $Ca^{++}$ on Transport and Uptake of $Sr^{85++}$ and $Ca^{45++}$ in Duodenal Segments in Experiment 1

| | $Sr^{85++}$ | | $Ca^{45++}$ | |
|---|---|---|---|---|
| *Variable* | *S/M*† | *G/M*† | *S/M*† | *G/M*† |
| Control | .85 (6) ±.09 | 1.5 (6) ±.16 | 2.8 (6) ±.82 | 4.5 (6) ±.82 |
| 0.010 M $MgCl_2$ | .43 (6) ±.02 | .76§ (6) ±.08 | 1.2 (6) ±.24 | 2.0 ‡ (6) ±.20 |
| 0.010 M $SrCl_2$ | .53 (6) ±.04 | .85‡ (6) ±.08 | 1.1 (6) ±.20 | 2.0 ‡ (6) ±.20 |
| 0.010 M $CaCl_2$ | .31‡ (7) ±.02 | .72§ (6) ±.06 | .40‡ (7) ±.03 | .99§ (6) ±.11 |

*Results expressed as mean (number of determinations) ± S.E.M. p values obtained by comparing control segments with all other segments.

†See text.

‡$p < 0.05$.

§$p < 0.01$.

**Table 3.** Inhibitory Effect of $Ca^{++}$, $Sr^{++}$, and $Mg^{++}$ on Transport and Uptake of $Ca^{45++}$, $Sr^{85++}$, and $Mg^{28++}$ in Duodenal and Ileal Segments

| | *Ratio calculated* | | | | | |
|---|---|---|---|---|---|---|
| | *G/M** | | | *S/M** | | |
| *Inhibitor* | $Ca^{45++}$ | $Sr^{85++}$ | $Mg^{28++}$ | $Ca^{45}$ | $Sr^{85}$ | $Mg^{28}$ |
| $Ca^{++}$ | | | | | | |
| Duodenum | ★ | ★ | + | ★ | ★ | o |
| Ileum | ★ | | ★ | ★ | | + |
| $Sr^{++}$ | | | | | | |
| Duodenum | ★ | ★ | + | + | + | o |
| Ileum | ★ | | + | o | | + |
| $Mg^{++}$ | | | | | | |
| Duodenum | ★ | ★ | ★ | + | + | o |
| Ileum | ★ | | ★ | o | | + |

*See text.

★ Inhibition significant statistically ($p < 0.05$).

\+ Inhibition, but $p > 0.05$.

o No inhibition.

Calcium was actively transported by the duodenum and the colon (Table 4), whereas magnesium was not concentrated in the serosal media by either the duodenum, jejunum, ileum, or colon. The total calcium and magnesium in both the serosal and mucosal media of all segments was greater at the end of the experiment than at the beginning. Obviously, this increase in calcium and magnesium was due to the re-

**Table 4.** Effect* of $Ca^{++}$, $Sr^{++}$ and $Mg^{++}$ on Transport and Uptake of $Ca^{45++}$ and $Mg^{28++}$ and on Transport of $Ca^{++}$ and $Mg^{++}$ in Duodenal and Ileal Segments

| Segment | Variable | $Mg^{28++}$ S/M† | $Mg^{++}$ S/M† | $Mg^{28++}$ G/M† | $Ca^{45++}$ S/M† | $Ca^{++}$ S/M† | $Ca^{45++}$ G/M† |
|---|---|---|---|---|---|---|---|
| Duodenum | Control | .44 (3) ±.03 | .95 (3) ±.07 | .93 (3) ±.03 | 1.3 (3) ±.35 | 1.1 (3) ±.17 | 1.4 (3) ±.11 |
| Duodenum | .033 M $CaCl_2$ | .45 (3) ±.03 | 1.3 ‡ (3) ±.06 | .85 (3) ±.05 | .49 (3) ±.04 | .45‡ (3) ±.04 | .73‡ (3) ±.12 |
| Duodenum | .033 M $SrCl_2$ | .49 (3) ±.05 | 1.5 (3) ±.23 | .77 (3) ±.09 | .62 (3) ±.10 | 1.2 (3) ±.17 | .85‡ (3) ±.20 |
| Duodenum | .033 M $MgCl_2$ | .57 (3) ±.04 | .56‡ (3) ±.04 | .41‖ (3) ±.01 | .69 (3) ±.04 | 1.5 (3) ±.23 | 1.1 (3) ±.11 |
| Jejunum | — | .59§ (3) ±.006 | 1.0 (3) ±.09 | 1.3 (3) ±.11 | .61 (3) ±.03 | 1.0 (3) ±.03 | 1.3 (3) ±.11 |
| Ileum | — | .63‡ (3) ±.03 | .80 (3) ±.04 | 1.7 § (3) ±.11 | .78 (3) ±.04 | .81 (3) ±.04 | 1.9 (3) ±.35 |
| Ileum | Control | .61 (4) ±.04 | 1.1 (4) ±.05 | 1.6 (4) ±.10 | .81 (4) ±.01 | 1.08 (4) ±.03 | 3.4 (4) ±0.1 |
| Ileum | .011 M $CaCl_2$ | .52 (4) ±.17 | 1.1 * (4) ±.15 | 1.1 § (4) ±.05 | .49§ (4) ±.06 | .57§ (4) ±.10 | 1.1 ‖ (4) ±.10 |
| Ileum | .011 M $SrCl_2$ | .47 (4) ±.06 | 1.1 (4) ±.35 | 1.3 (4) ±.15 | .80 (4) ±.04 | 1.1 (4) ±.15 | 1.8 § (4) ±.25 |
| Ileum | .011 M $MgCl_2$ | .51 (4) ±.05 | .53‡ (4) ±.04 | .83§ (4) ±.06 | .86 (4) ±.05 | .91 (4) ±.22 | 2.4 ‖ (4) ±.05 |
| Duodenum | — | .40 (3) ±.05 | 1.06 (3) ±.04 | .96§ (3) ±.06 | 1.6 ‡ (3) ±.23 | 1.8 ‡ (3) ±.17 | 3.3 (3) ±.23 |
| Colon | — | .41 (4) ±.04 | 1.18 (3) ±.08 | 1.6 (4) ±.20 | .96 (4) ±.13 | 1.4 ‡ (3) ±.06 | 4.0 (4) ±.70 |

*Results expressed as mean (number of determinations) ± S.E.M. p values obtained by comparing control duodenal segments with all other segments in the first 6 segments, and control ileal segments with all other segments in the last 6 segments.

†See text.

‡$<0.05$.

§$<0.01$.

‖$<0.001$.

lease of these cations by the intestines into the surrounding medium. The addition of $Ca^{++}$ to the duodenal mucosal medium increased the concentration of magnesium in the serosal medium, but the addition of $Mg^{++}$ to the duodenal mucosal medium did not increase the concentration of calcium in the serosal medium.

## Discussion

The studies of several investigators (*9–15*) indicate that there exists an inter-relationship in the pathways of absorption, secretion, and reabsorption in the gut, and in the renal tubular reabsorption of $Ca^{++}$, $Mg^{++}$, and $Sr^{++}$. Berglund and Forster found that the intravenous administration of $MgCl_2$ depressed calcium excretion in the urine of the aglomerular marine teleost, *Lophius americanus*, which secretes urine through its renal tubules. Conversely, they noted that the intravenous administration of $CaCl_2$ decreased magnesium excretion in the urine.

Alcock and MacIntryre found that rats on a low-magnesium diet excreted less calcium in the urine and feces than normals. The net absorption of calcium from the gut in the magnesium deficient rats—expressed in microequivalents—was equal to the sum of the calcium and magnesium absorbed by the normal group. Furthermore, they reported that rats on a low-calcium diet excreted less magnesium in the feces, and more magnesium in the urine. Cramer and Dueck (*16*) found in dogs that the absorption of calcium by isolated intestinal loops was decreased when magnesium chloride was added to the perfusate. Wasserman *et al.* (*17*) observed in rats that increased dietary calcium decreased the absorption of $Sr^{85++}$ and $Ca^{45++}$ effectively and to about an equal degree, although a four-fold increase in intake of $Sr^{++}$ did not reduce the absorption and retention of $Sr^{85++}$. In rats (*18, 19*), elevated dietary calcium decreases the ultimate body burden of radiostrontium.

In everted rat duodenal sacs it has been shown in vitro (*20*) that $Sr^{89++}$ and $Ca^{45++}$ can be transported against a concentration gradient, but $Sr^{89++}$ is much less actively transferred than is $Ca^{45++}$. In the present study, the ability to transport against a concentration gradient is regarded as indicating existence of active transport, and the marked increase in concentration within the gut tissue is regarded as indicating the existence of active uptake. However, because $Sr^{85++}$ is not as actively transported as $Ca^{45++}$, it is more convenient to measure

the *rate* at which $Sr^{85++}$ crosses the intestinal wall and the rate at which it is taken up.

Although 1 gm. of gut tissue may not occupy one milliliter, the distribution of $Ca^{45++}$ and $Sr^{85++}$ in the tissue and incubation medium is such that it is apparent from Table 1 that $Ca^{45++}$ and $Sr^{85++}$ are more concentrated in gut tissue than in the serosal medium.

In the case of $Ca^{45++}$ and $Sr^{85++}$, the rate of active uptake by the duodenal wall is significantly decreased in the presence of $Sr^{++}$, $Ca^{++}$, and $Mg^{++}$, and this is interpreted as representing a competitive relationship between Ca, Sr, and Mg ions for active uptake. These observations are comparable to those of Schachter and Rosen (*21*), who demonstrated in vitro that $Mg^{++}$ and $Co^{++}$ inhibited the ability of rat gut to concentrate $Ca^{45++}$, although $Sr^{++}$ and $Ba^{++}$ were without effect, and that the accumulation of $Ca^{45++}$ by slices of small intestines was decreased by $Mg^{++}$, $Sr^{++}$, and $Ba^{++}$.

$Ca^{++}$ significantly reduces the mean rate at which $Sr^{85++}$ crosses the duodenal wall. This is believed to be due to a competitive relationship between Ca and Sr ions for active transport. Paradoxically, $Sr^{++}$ itself is less effective than $Ca^{++}$ in reducing the rate of transport of $Sr^{85++}$ by the duodenum. Furthermore, the reduction in the mean rate of active transport of $Ca^{45++}$ in the presence of $Sr^{++}$ was not statistically significant. Also, $Sr^{85++}$ is actively taken up by the duodenal wall, and this is inhibited more by $Ca^{++}$ than $Sr^{++}$; the active uptake of $Ca^{45++}$ is inhibited more also by $Ca^{++}$ than by $Sr^{++}$. Thus it appears that there is competition between $Ca^{45++}$ and $Sr^{85++}$ for active uptake and active transport in the duodenal wall and that this tissue discriminates against $Sr^{85++}$ in preference to $Ca^{45++}$ for active uptake and active transport.

$Ca^{45++}$ is actively taken up by the duodenum, ileum, colon, and to a lesser degree by the jejunum. Nonradioactive calcium is actively transported by the duodenum and the colon. Schachter and Rosen have reported that $Ca^{45++}$ is actively transported by the proximal 15 cm. of the small intestine; the concentration gradients achieved were directly related to the proximity of the segment to the pylorus. D. D. Dziewiatkowski (personal communication to J. Z. H.) has found that the rat colon actively transports $Ca^{45++}$.

Ross (*22*) has proposed that magnesium is more efficiently transported in the ileum than in the jejunum. The fact that $Mg^{28++}$ is not concentrated from mucosal to serosal media by either the small or large intestines agrees with the findings of Ross for the small intestine.

The finding here, that the rate at which $Mg^{28++}$ crosses and is taken up by the intestinal wall is greatest at the distal ileum, less at the jejunum, and least at the duodenum, is consistent with an increase in absorptive efficiency for $Mg^{++}$ as the small intestine is descended.

$Ca^{++}$, $Sr^{++}$, and $Mg^{++}$ decrease the mean rate at which $Mg^{28++}$ crosses the ileum and the mean rate at which $Mg^{28++}$ is taken up by the ileum. However, only $Ca^{++}$ and $Mg^{++}$ significantly reduce the uptake of $Mg^{28++}$ by the ileum. These observations indicate that there is competition between $Ca^{++}$, $Sr^{++}$, and $Mg^{++}$ for uptake by the ileal wall. The addition of $Ca^{++}$ to the duodenal mucosal media increased the concentration of magnesium in the serosal media, but the addition of $Mg^{++}$ to the duodenal mucosal media did not increase the concentration of calcium in the serosal media. This observation suggests that $Ca^{++}$ has a greater affinity for duodenal tissue than has $Mg^{++}$.

It is conceivable that the gut's ability to increase the rate at which $Mg^{28++}$ crosses the intestinal wall and the rate at which $Mg^{28++}$ is taken up by the intestinal wall is due to an active transport mechanism, as has been shown for $Ca^{++}$ and $Sr^{++}$. Ross and Care (*23*) reported that normal intracellular accumulation of magnesium is dependent in part on energy derived from the oxidation of glucose. Ross (*22*) demonstrated in vitro that the relationship between increasing magnesium concentration and increasing magnesium transportation by the gut was not linearly proportional as might be expected in a purely passive process, but was diphasic. He suggested that there may be a saturating active process or a facilitated diffusion involved in the transport of magnesium. Thus $Mg^{28++}$ seems to be taken up and transported in a manner resembling both (*24*) facilitated transport and active transport.

In summary, the following observations have been made:

1. Either $Ca^{++}$, $Sr^{++}$, or $Mg^{++}$ significantly inhibit the active uptake of $Ca^{45++}$ and $Sr^{85++}$ by the duodenum.

2. $Ca^{++}$ significantly reduces the rate of active transport of $Sr^{85++}$ and $Ca^{45++}$.

3. However, $Sr^{++}$ is not as effective as $Ca^{++}$ in decreasing the rate of active transport of $Ca^{45++}$ and $Sr^{85++}$.

4. Furthermore, $Sr^{85++}$ is actively taken up by the duodenum, and this is inhibited more by $Ca^{++}$ than by $Sr^{++}$.

5. Also, the active uptake of $Ca^{45++}$ is inhibited more by $Ca^{++}$ than by $Sr^{++}$; consequently, these observations suggest that the duodenum

discriminates against $Sr^{++}$ in preference for $Ca^{++}$ in a competitive manner.

6. $Ca^{45++}$ is actively taken up by the duodenum, colon, and ileum and to a lesser degree by the jejunum.

7. The active uptake of $Ca^{45++}$ by the duodenum and ileum is inhibited by $Sr^{++}$ and $Mg^{++}$.

8. Calcium is actively transported by the duodenum and the colon.

9. $Mg^{28++}$ is actively taken up by the distal two-thirds of the small intestine and the colon.

10. The active uptake of $Mg^{28++}$ by the ileum is inhibited by $Ca^{++}$ but not significantly by $Sr^{++}$.

11. However, both $Sr^{++}$ and $Mg^{++}$ inhibit the active uptake of $Ca^{45++}$ by the ileum.

Therefore it is concluded that, at least in part, $Ca^{++}$, $Sr^{++}$, and $Mg^{++}$ absorption occurs by a common pathway in the gut. However, observed differences in the competitive effect of these three divalent cations in different parts of the G.I. tract suggest that the absorption of a particular cation may involve also other pathways.

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# A Mathematical Treatment of the Blood Dissociation Curve for Oxygen

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**A quantitative description of Bohr's effect can be made from the oxygen dissociation curves of hemoglobin at different pHs, and the dissociation constants of the $O_2$-linked acidic group of Hb ($K_R$) and $HbO_2$ ($K_O$) have been calculated as, respectively, $1.29\ 10^{-8}$ and $3.42\ 10^{-7}$ at 37°.**

**On the assumption that the oxygenation of hemoglobin takes place in four successive steps, the constant for each equilibrium can easily be calculated, and the values given. It appears that the oxygenation takes place with the same affinity for the first three steps, while the affinity for the fourth oxygenation is 125 times greater.**

**On the basis of these results a simplification of Adair's formula is given, containing only two constants, one ($K$) being representative of the affinity of the oxygen for the first three hemes, and the other ($m$) being the increase of affinity for the fourth oxygenation. This formula seems to fit most data in the literature of hemoglobin solutions and of blood in vivo and in vitro.**

**The physiological effects and the functional importance of the increased affinity for the fourth oxygenation are described, and the possibility that disturbances of the Hb oxygenation process may be due to the lack of this process is considered.**

THE oxygen dissociation curve of hemoglobin is certainly one of the most representative elements in the description of the respiratory function of the blood and, for this reason, it has been extensively studied. Its mathematical analysis, however, is made difficult because of the many factors that affect the fundamental reaction of combination of oxygen with hemoglobin:

1. The combination of Hb with oxygen is not a simple bimolecular reaction, but the resultant of four successive reactions, as pointed out in 1923 by Adair (*2*). Hemoglobin exists in blood as a tetrapolymer ($Hb_4$) and a combination of $O_2$ with each Hb unit must be a four-step reaction.

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2. The combination of Hb with $O_2$ is facilitated by the dissociation of a particular, so-called, $O_2$-linked acidic group. In alkaline solution, this is dissociated and oxygen has more affinity for Hb. This increased affinity of Hb for $O_2$ in an alkaline medium may be described as a higher tendency to dissociation of such an acidic group when Hb binds with $O_2$. This is the well-known Bohr effect, described in 1904.

3. The affinity of Hb for $O_2$ does not change only as a function of pH, but also as a function of $CO_2$ in solution. Margaria and Green reported in 1933 that $CO_2$ decreases the affinity of Hb for $O_2$, independently of any change of the pH or ionic strength of the solutions. This effect is due, in part at least, to the formation of a carbamino compound that is known to take place at high pH values.

4. The affinity of Hb for $O_2$ is decreased when electrolytes other than NaCl, such as phosphate and lactate, are present in solution.

5. Different gases, such as CO, which combine with Hb, may be present and, even when in small amounts, interfere substantially with the combination of Hb with $O_2$.

Besides these, other factors, generally easier to control, such as temperature, ionic strength, dilution of Hb, may affect the shape of the oxygen dissociation curve of hemoglobin.

To reach a better understanding of the reaction of the binding of $O_2$ to Hb, the number of variables that may affect such a reaction must be reduced as far as possible; only then may a quantitative treatment and an accurate description of the phenomenon be possible. The factors indicated in Paragraphs 3, 4, and 5 above do not come into play if the hemoglobin solutions used are pure and the only electrolyte is NaCl, and these are equilibrated with mixtures of oxygen and nitrogen absolutely free of other gases.

By changing only the $[H^+]$ in such solutions the Bohr effect can be studied quantitatively. It was thought that this effect should be mathematically defined before attempting the quantitative study of the reaction of combination of Hb with $O_2$, as described in Paragraph 1 above.

## The Dissociation Constants of the Oxygen-Linked Acidic Group of Hb and $HbO_2$

Let us assume that the dissociation of the $O_2$-linked group is the same for all Hb units of the $Hb_4$ molecule. Then this dissociation can be expressed by the equilibria

$$Hb \rightleftharpoons Hb^- + H^+ \qquad (K_R) \tag{1}$$

$$HbO_2 \rightleftharpoons HbO^-_2 + H^+ \qquad (K_O) \tag{2}$$

where $K_R$ and $K_O$ are the equilibrium constants.

The reaction of Hb with $O_2$ may be taken as represented by

$$Hb + O_2 \rightleftharpoons HbO_2 \qquad (K_{O_2}) \tag{3}$$

$$Hb^- + O_2 \rightleftharpoons HbO^-_2 \qquad (K'_{O_2}) \tag{4}$$

the equilibrium constants being indicated on the sides. In such equilibria any difference between the first, second, third, and fourth oxygenation process is temporarily disregarded.

If the law of mass action is applied to the Reactions 1, 2, 3, and 4, the following equations are obtained:

$$\frac{[Hb^-]}{[Hb]} = \frac{K_R}{[H^+]} \tag{5}$$

$$\frac{[HbO^-_2]}{[HbO_2]} = \frac{K_O}{[H^+]} \tag{6}$$

$$\frac{[HbO_2]}{[Hb]} = K_{O_2}P_{O_2} \tag{7}$$

$$\frac{[HbO^-_2]}{[Hb^-]} = K'_{O_2}P_{O_2} \tag{8}$$

and, adding 1 to both members of Equations 5 and 6:

$$\frac{[Hb^-] + [Hb]}{[Hb]} = \frac{K_R + [H^+]}{[H^+]} \tag{9}$$

$$\frac{[HbO^-_2] + [HbO_2]}{[HbO_2]} = \frac{K_O + [H^+]}{[H^+]} \tag{10}$$

Then, rearranging Equations 7, 9, and 10:

$$\frac{[HbO^-_2] + [HbO_2]}{[Hb^-] + [Hb]} = K_{O_2}P_{O_2}\frac{K_O + [H^+]}{K_R + [H^+]} \tag{11}$$

or, in logarithmic form,

$$\log P_{O_2} = pK_{O_2} + \log \frac{[HbO^-_2] + [HbO_2]}{[Hb^-] + [Hb]} + \log \frac{K_R + [H^+]}{K_O + [H^+]} \tag{12}$$

The $K_{O_2}$ value, as calculated, may be different at the various saturation levels if the four constants for the four successive steps of oxygenation are different. For a given saturation value, however, this is assumed to be constant. Then, by differentiating Equation 12:

$$-\frac{\log P_{O_2}}{pH} = \frac{[H^+]}{[H^+]+K_R} - \frac{[H^+]}{[H^+]+K_0} = \frac{[H^+]\,(K_0 - K_R)}{(K_0+[H^+])\,(K_R+[H^+])} \qquad (13)$$

By knowing the $P_{O_2}$ and pH of the solution, for a given saturation value of Hb such as in the experiments described in Fig. 1, Equation 13 can be defined quantitatively and $K_0$ and $K_R$ calculated.

In fact, from the data of Fig. 1, $P_{O_2}$ or log $P_{O_2}$ can be plotted as a function of pH at the different saturation values (Fig. 2) and from this, $\frac{\delta \log P_{O_2}}{\delta\, pH}$ read for any $[H^+]$ value, to give a numerical value in Equation 13.

Figure 2 shows that all the curves obtained at different saturations are parallel, namely that $\frac{\delta \log P_{O_2}}{\delta\, pH}$ is the same for all saturation values. This confirms the validity of the assumption made above, namely that $K_0$ and $K_R$ are the same for all Hb units of the hemoglobin molecule. If this were not the case, $\frac{\delta \log P_{O_2}}{\delta\, pH}$ would change with changing saturation, i.e. with changing the Hb unit involved. The calculated values $K_0$ and $K_R$ are given in Table 1.

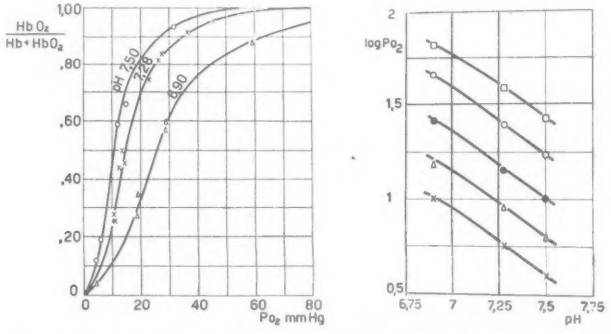


**Fig. 1** (*left*). Dissociation curve of horse Hb in concentration 2.35 mM/L. (4 gm./100 ml.) at the pH values indicated. The temperature was 37°, the ion strength (NaCl) 150 mM/L. From Margaria *et al.* (*15*). **Fig. 2** (*right*). Log $P_{O_2}$ as a function of pH for $S = 0.01$, 0.02, 0.05, 0.08, 0.09, as from data of Fig. 1. From Margaria *et al.* (*15*).

**Table 1.** Dissociation Constants of $O_2$-Linked Acid Group of Hb ($K_R$) and $HbO_2$ ($K_0$)

| *Author* | $K_0$ | $K_R$ | °C. |
|---|---|---|---|
| Margaria *et al.* (*15*) | $3.42 \times 10^{-7}$ | $1.29 \times 10^{-8}$ | 37 |
| Wyman & Ingalls (*6*)* | $2.09 \times 10^{-7}$ | $1.18 \times 10^{-8}$ | 25 |
| Hastings *et al.* (*3*)* | $2.4 \times 10^{-7}$ | $0.66 \times 10^{-8}$ | 38 |

*Data given for comparison.

Such values may be obtained also from the difference of the titration curves of reduced and oxygenated Hb; in fact the dissociation of the $O_2$-linked acidic group of Hb, all other acidic groups being neglected, is defined (for Hb as $HbO_2$ at a concentration equal to 1) by the familiar formula common to all acids:

$$B_{Hb} = \frac{K_R}{K_R + [H^+]} \tag{14}$$

$$B_{HbO_2} = \frac{K_O}{K_O + [H^+]} \tag{15}$$

These are described graphically in Fig. 3, $B$ being the mole of base per mole of Hb or $HbO_2$, added to the solution, which is equivalent to the mole concentration of the dissociated protein.

The difference between the two curves at any pH value is then:

$$B_{HbO^-_2} - B_{Hb} = \left|\frac{\delta B}{\delta HbO_2}\right|_{pH} = \frac{[H^+]\,(K_O - K_R)}{(K_O + [H^+])\,(K_R + [H^+])} \tag{16}$$

The expression at the right is the same as for Equation 13; therefore

$$-\frac{d \log P_{O_2}}{d\,pH} = \left|\frac{\delta\ B}{\delta\ HbO_2}\right|_{pH} \tag{17}$$

The same conclusion was reached by Wyman in 1948 (*7*) through a different, more elaborate procedure. This indicates that the two procedures to calculate $K_R$ and $K_O$, one based on the difference of the titration curves of Hb and $HbO_2$ (*3, 6*), and the other based on the effect of pH on the shift of the dissociation curve for CO (*15*) though substantially different, are equally applicable.

It follows necessarily from Equation 17 that $\frac{\delta B}{\delta HbO_2}$ and, from Equation 16, $K_R$ and $K_O$, are independent from saturation. In other words, the acidic dissociation constants of the $O_2$-linked group are the same

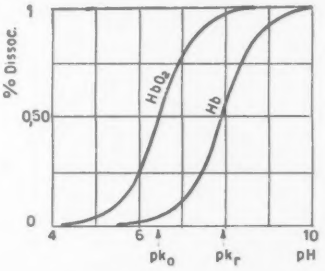


**Fig. 3.** Dissociation curves of $O_2$-linked group of Hb calculated from Equations 14 and 15, $K_O$ and $K_R$ being given values shown in Table 1.

for the 4 units in $Hb_4$, and the acidity due to the oxygenation of the hemoglobin is therefore the same at all saturation values. The same conclusion was reached in 1924 by Hastings *et al.* (*3*), who found, at different saturation values of hemoglobin with oxygen, the same $\frac{\delta B}{\delta HbO_2}$ value.

Previous results obtained at our laboratory in 1955 (*12, 13*) seemed to lead to the conclusion that, at very low or at very high saturation values, the acidity due to oxygenation was higher than at intermediate values. However, we later showed such an increase to be only apparent (*15*). The data given here confirm that the Bohr effect is the same at all saturation values of hemoglobin with oxygen.

### Bohr Effect

It is clear that the Bohr effect, as described from Equation 12 or, better, from Equations 13 and 16, is quantitatively defined when $K_O$ and $K_R$ are known. From Equation 16 and from the data given in Table 1, Fig. 4 has been drawn. It is evident that the Bohr effect is maximum at a pH of 0.5 ($pK_O + pK_R$), and that it tends towards zero at very high $[H^+]$ when both $B_{Hb}$ and $B_{HbO_2}$ tend toward zero (Equations 14 and 15) or at very low $[H^+]$ where both these factors tend towards 1.

The maximum $\frac{\delta B}{\delta HbO_2}$ value appears from this calculation to be equal to 0.67, which is the same as obtained by Hastings *et al.* (*3*) from titration curves of reduced and oxygenated Hb.

The considerations above do not take into account the possible other $O_2$-linked acidic groups that may be present in other ranges of pH, such as one described by Wyman (*7*) at pH 5.

### The Reaction of Hb with $O_2$

It was previously mentioned that the hydrogen of the $O_2$-linked carboxyl group of Hb competes with $O_2$. When it is absent, namely when

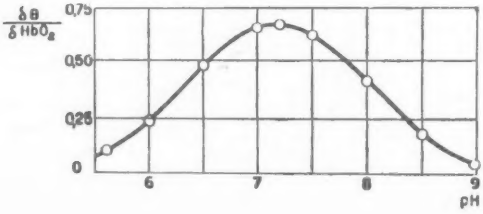


**Fig. 4.** Acidifying effect of oxygenation of hemoglobin as a function of pH, calculated from Equation 16 from data given in Fig. 3.

the hemoglobin is dissociated, the binding of hemoglobin with oxygen is facilitated. This is the reason why the equilibrium given in Equation 4 is displaced to the right more than that given in Equation 3, and why $K'_{O_2}$ is greater than $K_{O_2}$. These two constants however are simply related by a coefficient of proportionality which is the ratio of the two acidic dissociation constants of the $O_2$-linked group for $HbO_2$ and for Hb.

In fact, by applying the law of mass action to Equilibria 1–4 and rearranging, the following equation is derived:

$$\frac{K_{O_2}}{K'_{O_2}} = \frac{K_R}{K_O} \tag{18}$$

As $K_R$ and $K_O$ values are the same for the 4 Hb units of the hemoglobin molecule, the ratio $\frac{K_{O_2}}{K'_{O_2}}$ will always be the same, even if the absolute values of the constants are different for the 4 Hb units. From Equation 11, the saturation of Hb, the $P_{O_2}$, and the pH of the solution being known, the equilibrium constant $K_{O_2}$ of the reaction of the oxygenation of the hemoglobin may be calculated. This constant applies to the condition of very high acidity of the solution, where $K_O$ and $K_R$ are negligible, relative to $[H^+]$. Equation 11 may be used also to correct the value obtained for the constant of oxygenation at any $[H^+]$ to obtain the $K_{O_2}$ value, as though only Reaction 3 would take place. If such a correction is made on all the data on which the three curves of Fig. 1 have been drawn, a single curve would describe the process, as shown in Fig. 5.

The process described in Fig. 5 is now much simpler as it describes Reaction 3 alone, without the interference of Reactions 1, 2, and 4.

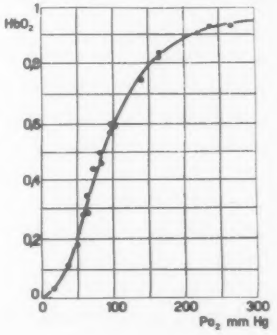


**Fig. 5.** Dissociation curve of Hb for oxygen, from data in Fig. 1 corrected by use of Equation 11 for $[H^+] = \infty$.

The process of Reaction 3 can therefore be treated quantitatively, considering that the oxygenation reaction takes place in four successive steps:

$$Hb_4 + O_2 \rightleftharpoons Hb_4O_2 \qquad (K_1) \qquad (19)$$

$$Hb_4O_2 + O_2 \rightleftharpoons Hb_4O_4 \qquad (K_2) \qquad (20)$$

$$Hb_4O_4 + O_2 \rightleftharpoons Hb_4O_6 \qquad (K_3) \qquad (21)$$

$$Hb_4O_6 + O_2 \rightleftharpoons Hb_4O_8 \qquad (K_4) \qquad (22)$$

the equilibrium constant being indicated for each equilibrium. From these Adair obtained the well-known formulation

$$S = \frac{K_1P_{O_2} + 2K_1K_2P_{O_2}^2 + 3K_1K_2K_3P_{O_2}^3 + 4K_1K_2K_3K_4P_{O_2}^4}{4(1 + K_1P_{O_2} + K_1K_2P_{O_2}^2 + K_1K_2K_3P_{O_2}^3 + K_1K_2K_3K_4P_{O_2}^4)} \qquad (23)$$

where $S$ is the fraction of the hemoglobin saturated with $O_2$.

The values of the constants $K_1$ to $K_4$ can be obtained by applying the method of minimum squares to this equation; this has been done by Roughton *et al.* (*11*) with the aid of an electronic computer. They used 3–4 gm./100 ml. sheep hemoglobin solutions at pH 9.1 and 7.1 in two sets of experiments. The values of the constants so calculated in the different experiments differed widely in spite of the fact that all experiments were performed on animals of the same species. Furthermore $K_3$ and $K_4$, as calculated from an experiment performed at pH 7.1, turned out to have negative values. This result does not seem reasonable.

A peculiarity of these experiments was that $K_4$, as calculated, had a value consistently greater (from 3 to 100 times) than the first three $K$s. A nonconsistent relationship was found among the first three constants.

Fenne and Nahas (*14*) made the same calculation from an oxygen dissociation curve of human blood in vivo obtained from data by Lambertsen *et al.* (*10*). Calculation for the three constants, $K_1$, $K_2$, and $K_4$ resulted in negative values, a finding that the authors attribute either to small errors in the determination of $P_{O_2}$ and/or $S$ or to a mechanism of Hb oxygenation different from that postulated by Adair.

The results obtained by this method are not very encouraging and this may be due to the inadequacy of the method. When the matrix of the coefficient of a normal system, i.e., the final resolutive system for the values of the $K$s, is critical and its determined value is approaching

zero, the method of minimum squares is no longer applicable. At this point even extremely small deviation of the $P_{O_2}$ and $S$ values may result in substantial absolute errors of the $K$ as calculated (*20, 21*).

On the other side, the mechanism described by Adair, as based on the four successive reactions of oxygenation of Hb, has been questioned recently (*17, 19*), and this particularly on the basis of the inconsistency of the values for the equilibrium constants as found by different authors.

Assuming as valid the hypothesis of the four successive reactions of oxygenation of Hb, as expressed by Adair's equation for the condition of equilibrium, a calculation of the 4 oxygenation constants may be made by calculating the successive constants one at a time, using a simplified version of Adair's formula.

At low saturation values the first reaction is predominant and Reactions 20–22 may be neglected. Equation 23 then takes the form:

$$S = \frac{K_1 P_{O_2}}{4(1 + K_1 P_{O_2})} \tag{24}$$

This formula has been used by Paul and Roughton (*9*) for experimental values of $S < 0.01$, and pH 9.1, so that only Reaction 4 was involved. Such a method, however, implies an extreme precision of determining very low values of $S$ and $P_{O_2}$, where even small errors have a great relative importance. Furthermore the pH of the solution was very far from the actual pH in vivo.

Margaria *et al.* (*15, 16*) have used this formulation to calculate the $K_1$ values at different saturation values, from 0.05 to 0.20, and by extrapolating the obtained values to $S$ or $P_{O_2} = 0$, to obtain the correct value of $K_1$.

A better extrapolated value of $K_1$ is obtained if the first two equilibria are considered as from

$$S = \frac{K_1 P_{O_2} + 2K_1 K_2 P_{O_2}^2}{4(1 + K_1 P_{O_2} + K_1 K_2 P_{O_2}^2)} \tag{25}$$

to $K_2$ being given an approximate value. Plotting, then, $K_1$, or $\log K_1$, as a function of both $P_{O_2}$ and $S$, as in Fig. 6, a reliable value of $K_1$ is obtained.

The constant $K_4$ may be obtained with a similar procedure, considering the deoxygenation of the hemoglobin—namely, considering first

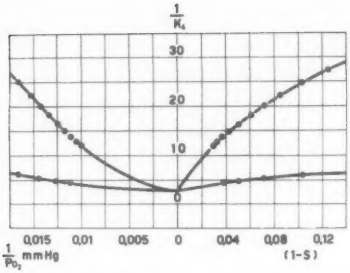


**Fig. 6.** Values of $\frac{1}{K_4}$, using Equation 29 (upper two curves) and Equation 30 (lower two curves), as calculated from experimental data, plotted both as function of $\frac{1}{P_{O_2}}$ (left), and $1 - S$ (right). From Margaria *et al.* (*20*).

Reaction 22 and then successively 21, 20, and 19. A formulation similar to Adair's is then obtained:

$$1 - S = \frac{\dfrac{1}{K_4 P_{O_2}} + \dfrac{2}{K_4 K_3 P_{O_2}^{\,2}} + \dfrac{3}{K_4 K_3 K_2 P_{O_2}^{\,3}} + \dfrac{4}{K_4 K_3 K_2 K_1 P_{O_2}^{\,4}}}{4\left(1 + \dfrac{1}{K_4 P_{O_2}} + \dfrac{1}{K_4 K_3 P_{O_2}^{\,2}} + \dfrac{1}{K_4 K_3 K_1 P_{O_2}^{\,3}} + \dfrac{1}{K_4 K_3 K_2 K_1 P_{O_2}^{\,4}}\right)} \qquad (26)$$

By simplifying this equation to the first equilibria to obtain an equation similar to 24 or to 25, and proceeding as explained to calculate $K_1$, the value of $K_4$ can be obtained.

$K_1$ being known, $K_2$ may be obtained with Equation 25, and similarly, $K_4$ being known, $K_3$ can be obtained with a corresponding formulation.

Alternatively $K_2$ and $K_3$ may be obtained more simply with:

$$\underbrace{\frac{K_1 P_{O_2}^{\,2}\,(2 - 4S)}{4S + K_1 P_{O_2}\,(4S - 1)}}_{y} = \frac{1}{K_2} + K_3 \underbrace{\frac{K_1 P_{O_2}^{\,3}\,(4S - 3) + K_1 K_4 P_{O_2}^{\,4}\,(4S - 4)}{4S + K_1 P_{O_2}\,(4S - 1)}}_{x} \qquad (27)$$

which is a rearrangement of Adair's formula. By giving to $y$ and to $x$ the values as indicated, Equation 27 is that of a straight line, and $K_2$ and $K_3$ can be easily determined graphically.

More recently, instead of Equation 24, Margaria *et al.* (*20*) have found it more profitable to use a Taylor's series expansion of Adair's formula:

$$S = \frac{1}{4} K_1 P_{O_2} + \ldots . \qquad (28)$$

$K_1$ is then calculated on data obtained at saturation values lower than 25%; $K_4$ can be calculated with similar procedure, the corresponding Taylor's expansion being:

$$1 - S = \frac{1}{4 K_4 P_{O_2}} + \ldots . \tag{29}$$

or, taking into account also the second deoxygenation reaction:

$$1 - S = \frac{1}{4 K_4 P_{O_2}} + \left( \frac{1}{2 K_3 K_4} - \frac{1}{4 K_4^2} \right) \frac{1}{P_{O_2}^2} - \ldots . \tag{30}$$

in which an approximate value is given to $K_3$. The last equation is particularly convenient for calculating $\frac{1}{K_4}$, as $\frac{1}{K_3}$ is much bigger, and, if this second reaction is completely neglected, the calculated value of $K_4$ may be affected by a very appreciable error.

When all the experimental data are in the upper range of saturation, which is the case of Lambertsen's data obtained in vivo, $K_4$ and $K_3$ are first calculated in succession, and $K_1$ and $K_2$ are calculated with the equation:

$$\underbrace{\frac{P_{O_2}(1 - 4S)}{4S}}_{y'} = \frac{1}{K_1} + \underbrace{K_2 P_{O_2}^2 \left(1 + K_3 P_{O_2} + K_3 K_4 P_{O_2}^2 - \frac{2 + 3 K_3 P_{O_2} + 4 K_3 K_4 P_{O_2}^2}{4S}\right)}_{x'} \tag{31}$$

which again is a rearrangement of Adair's equation similar to Equation 27.

The values of the constants calculated with this method on Lambertsen's (*10*) Dill's (*22*), and Milla's (*23*) data at pH 7.4 are reported in Table 2.

When these values are substituted in Equation 23, three functions, whose graphs approach most satisfactorily the experimental curves, are obtained.

**Table 2.** Values of Constants Calculated on Data from Various Authors at pH 7.4

| *Author* | *Substrate* | $K_1$ | $K_2$ | $K_3$ | $K_4$ |
|---|---|---|---|---|---|
| Lambertsen *et al.* (*10*) | Human blood (in vivo) | 0.066 | 0.018 | 0.010 | 0.36 |
| Dill (*22*) | Human blood (in vitro) | 0.040 | 0.029 | 0.0065 | 0.40 |
| Milla (*23*) | Horse hemoglobin (in vitro) | 0.050 | 0.020 | 0.0078 | 0.48 |

### Interaction Among the Hemes

If the affinity for $O_2$ were the same for all 4 hemes, the reaction of oxygenation could be represented, instead of the 4 successive reactions of $Hb_4$, with one molecule of $O_2$ at a time, by the simple equilibrium

$$Hb + O_2 \rightleftharpoons HbO_2$$

where Hb is the fundamental unit, and $[Hb] = 4[Hb_4]$; the corresponding dissociation curve would be described simply by the hyperbola:

$$S = \frac{K P_{O_2}}{1 + K P_{O_2}} \qquad (32)$$

$K$ being the equilibrium constant of this reaction.

Hemoglobin in blood, however, is not in the form of Hb but of $Hb_4$, and Equation 23 must apply. This equation can be reduced to the form of Equation 32 if the constants $K_1$, $K_2$, $K_3$, and $K_4$ have the values given in Table 3.

**Table 3.** Values of Constants $K_1$ to $K_4$ of Equation 23 to Fit Equation 32 Calculated in Table 2

| | | |
|---|---|---|
| $K_1$ | = | $4\ K$ |
| $K_2$ | = | $1.5\ K$ |
| $K_3$ | = | $0.66\ K$ |
| $K_4$ | = | $0.25\ K$ |

The values of the first three constants, as calculated and given in Table 2, are approximately in the ratio indicated in Table 3. This is evidence that the affinity for oxygen is the same for the first three hemes. This conclusion was reached on all the data tested, as reported in Table 2.

The same finding has been obtained also on Roughton's data (*11*) on sheep hemoglobin in absence of $CO_2$ at pH 7.1.

The value of the fourth constant, on the contrary, is approximately 125 times that expected from the data in Table 3. This seems to indicate that the oxygenation of the last heme is facilitated by the presence of three molecules of $O_2$ in the other three hemes—i.e., that there is interaction limited to the fourth $O_2$ binding.

If there is no interaction, the values of the four constants as calculated, divided by the coefficient indicated in Table 3, should give the same value. Table 4 shows that this is the case only for the first three constants. The average $K$ obtained for these is also given. The in-

crease, $m$, of the $K$ value as calculated from $K_4$, over the average $K$ value, calculated from $K_1$, $K_2$, $K_3$ is also indicated.

The values of $K_1$, $K_2$, $K_3$, and $K_4$, in Equation 23 can now be replaced by their value, as a function of $K$ and $m$, and a simplified equation obtained:

$$S = \frac{\left(\frac{1+KP_{O_2}}{KP_{O_2}}\right)^3 + m - 1}{\left(\frac{1+KP_{O_2}}{KP_{O_2}}\right)^4 + m - 1} \qquad (33)$$

By giving to $K$ and to $m$ the numerical values calculated on each set of experimental data of Dill (*22*), Lambertsen (*10*), and Milla (*23*), the curve, as described by Equation 33, fits the experimental data satisfactorily, as shown for Dill's data in Fig. 7.

A formulation substantially similar to Equation 33 has been worked out by Roughton (*8*) on the same assumptions. His experimental data

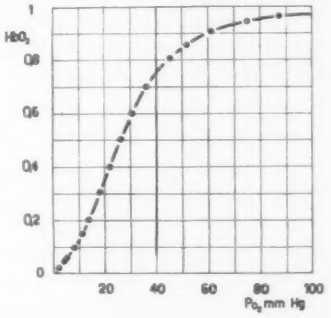


**Fig. 7.** Dissociation curve of blood in vitro calculated with Equation 33 and constants given in Table 3 from experimental data of Dill (*22*). From Margaria *et al.* (*20*).

**Table 4.** Values for Four Constants Divided by Coefficients in Table 3

| *Source of data* | *pH* | $K = 1/4\ K_1$ | $K = 2/3\ K_2$ | $K = 3/2\ K_3$ | $K = 4\ K_4$ | *Average K for values of 1st 3 columns* | $m = \frac{K_4}{1/4\ K}$ (*Mean*) |
|---|---|---|---|---|---|---|---|
| Dill (*22*) | 7.4 | 0.0100 | 0.0193 | 0.0097 | 1.600 | 0.0130 | 123 |
| Lambertsen *et al.* (*10*) | 7.4 | 0.0160 | 0.0120 | 0.0150 | 1.440 | 0.0144 | 100 |
| Milla (*23*) | 7.4 | 0.0125 | 0.0133 | 0.0117 | 1.920 | 0.0125 | 154 |
| Roughton *et al.* (*11*)* | 7.1 | 0.0157 | 0.0165 | 0.0225 | 2.322 | 0.0182 | 127 |

*Values obtained on sheep hemoglobin solutions in absence of $CO_2$.

fit the equation satisfactorily, and $S$, as calculated, does not differ from the experimental values by more than $\pm$ 0.015. He found however, that it is possible to fit the experimental data with the same accuracy with different sets of $K_1$, $K_2$, $K_3$, $K_4$ values. The conclusion was reached therefore that more accurate data were required to calculate reliable values of such constants and to obtain a satisfactory answer to the problem of interaction of the first three hemes.

Roughton's later (*11*), more accurate data seem to favor the hypothesis of a heme-heme interaction even in the first three oxygenation processes. The pH of his Hb solutions however was very high, and this method for the calculation of all four constants simultaneously may not be appropriate. A separate calculation of the single constants, as described in this paper, may give more consistent results.

### The Equilibrium Constants for Reactions 19–22

The data obtained by Milla (*23*) on horse hemoglobin solutions in absence of $CO_2$ do not contain the error due to the interference of this substance in the process of oxygenation. Therefore the constants of Equilibria 19–22, valid for the undissociated hemoglobin (Reaction 3) may be calculated from the data of Table 3 with the aid of Equation 11. Such values are:

$$K_1 = 0.00945$$

$$K_2 = 0.00365$$

$$K_3 = 0.00160$$

$$K_4 = 0.08450$$

From this it appears in fact that any oxygenation constant of Hb at any given $[H^+]$, $K'_{O_2}$ is

$$K_{O_2}{}' = K_{O_2} \frac{K_0 + [H^+]}{K_R + [H^+]} \tag{34}$$

for which, $[H^+]$ being known, $K_{O_2}$ is calculated.

### Definition of the $HbO_2$ Dissociation Curve from the $P_{O_2}$ Value at Semisaturation

Giving to $K$ the value 0.0133, which is the mean obtained from Lambertsen's (*10*), Dill's (*22*), and Milla's (*23*), data at pH 7.4, and giving to $m$ the average value, as found, of 125, and substituting in Equation 33, a function is obtained which describes with the best approximation all the dissociation curves considered for blood and Hb solutions. The value of $P_{O_2}$ at $S = 0.5$, so calculated, differs by no more than $\pm$ 0.5 mm.

Hg from that indicated by the curve as drawn with the best approximation through all the experimental points.

If, in Equation 33, $m$ is set at 125, a value which appears to be valid for the conditions of ionic strength, Hb concentration, and pH of the

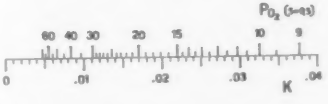


**Fig. 8.** Nomogram for reading value of $K$ when $P_{O_2}$ is given at half saturation, using Equation 33, with $m = 125$. From Margaria *et al.* (*20*).

blood, the value of $K$ is defined if, for any given $S$ value, $P_{O_2}$ is known. The nomogram of Fig. 8 was calculated so as to give directly the value of $K$ for any $P_{O_2}$ value at half saturation. The effects of different factors (temperature, pH, $P_{CO_2}$ etc.) on the oxygen dissociation curve can therefore be expressed simply as a variation of $K$, and thus they are more susceptible to a quantitative approach.

### The Bohr Effect as Described by a Variation of the Constant $K$ of Equation 33

According to the data existing in the literature, in a reasonable range of pH (5–8.5), the Bohr effect does not appear to involve a change of the shape of the $O_2$ dissociation curve, and, if saturation is plotted against log $P_{O_2}$ instead of $P_{O_2}$, parallel curves at different pH values are obtained if the ionic strength of the solution is kept constant. In other words, the effect of pH is the same at low as at high oxygenation of the hemoglobin, and this means that the four oxygenation reactions are affected to the same extent by a pH change. The four equilibrium constants, $K_1$–$K_4$, change therefore proportionately (*7*, *15*), which implies that the factor $m$ of Equation 14 is independent of pH.

The oxygenation constants $K_1$, $K_2$, $K_3$, $K_4$, or $K$ of Equation 33 change with changing $[H^+]$ by a factor of 26.5, from very low pH, where it is approximately $\frac{K_0 + [H^+]}{K_R + [H^+]} = 1$ to very high pH, where it is approximately

$$\frac{K_0 + [H^+]}{K_R + [H^+]} = \frac{K_0}{K_R} = \frac{34.2 \times 10^{-8}}{1.29 \times 10^{-8}} \tag{35}$$

The change is 6-fold for a pH shift from 6.4 to 8.0.

### Effect of the Affinity Increase of the Fourth Oxygenation Reaction

The effect of the increase of the affinity of the fourth oxygen molecule for the Hb on the $O_2$ dissociation curve is evident from the curves

in Fig. 9, which are the graphical representations of Equations 32 and 33, $K$ being given the same value in both cases. It is evident that the increase of the affinity for the fourth oxygenation is sufficient to give to the $O_2$ dissociation curve the characteristic S-shape, and its effect

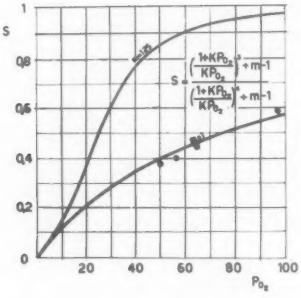


**Fig. 9.** Dissociation curve of $HbO_2$ according to equation indicated, with $m = 125$ (upper curve) and $m = 1$ (lower curve); for both curves $K = 0.025$. Circles indicate experimental data obtained in vivo by Reissmann *et al.* (*18*) on a case of a familiar human hemoglobin variant which seems characterized by absence of heme-heme interaction.

increases by increasing the saturation. The value of $m$ being fairly high, its influence is already appreciable at very low $P_{O_2}$.

Thanks to this arrangement, the saturation of the Hb with $O_2$ can be almost complete in normal life conditions. In fact, a value of $S = 0.98$ is reached at normal alveolar $P_{O_2}$ values which would otherwise be reached at 4000 mm. Hg.

Interesting in this connection is the case of a familiar human hemoglobin variant described by Reissmann *et al.* (*18*), which was characterized by a very low oxygen saturation at the normal arterial oxygen tension of the blood. The author came to the conclusion that, while the Bohr effect was of normal magnitude, "probably no heme-heme interaction" took place in this hemoglobin molecule. The experimental data obtained by Reissmann *et al.* in vivo have been plotted in Fig. 9. They fit remarkably well with the curve as calculated on the assumption of no interaction between the hemes, thus confirming the pathogenetic mechanism advanced by the authors.

### Fractional Representation of the Different Hb Compounds

The values of $K_1$, $K_2$, $K_3$, and $K_4$ being defined, it is possible to calculate at all saturation values the single fractions of $[Hb_4]$, $[Hb_4O_2]$, $[Hb_4O_4]$, $[Hb_4O_6]$, and $[Hb_4O_8]$.

In fact, from the formula expressing the mass-action law for each single equilibrium, setting $[Hb_4] = 1$

$$[Hb_4O_2] = K_1P_{O_2} \qquad [Hb_4O_6] = K_1K_2K_3K_4{P_{O_2}}^3$$

$$[Hb_4O_4] = K_1K_2{P_{O_2}}^2 \qquad [Hb_4O_8] = K_1K_2K_3K_4{P_{O_2}}^4$$

From these, the fractions of each component are easily calculated, and the values given graphically (Fig. 10). The great increase in

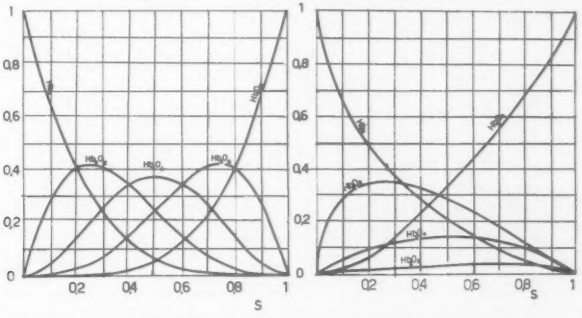


**Fig. 10.** Fractions of $Hb_4$, $Hb_4O_2$, $Hb_4O_4$, $Hb_4O_6$, and $Hb_4O_8$, as a function of saturation, as calculated assuming (left) that no interaction takes place among the 5 hemes and (right) that no interaction exists for the first three hemes, but that the oxygenation of the fourth takes place with an affinity for $O_2$ 125 times greater than that of the first ones, which is the actual case for normal blood.

affinity of [Hb] for the fourth oxygen molecule is illustrated by the preponderance of $[Hb_4O_8]$ over $[Hb_4O_6]$ which is reduced to negligible values at any value of $S$, as compared with the amount present as calculated from the assumption that $m = 1$.

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# A Rapid Procedure for Catalase Determination in Blood and Tissue Samples with the Van Slyke Manometric Apparatus

*John Esben Kirk*

**A precise and rapid gasometric procedure is described for catalase determination in blood and tissue samples. The enzyme activity assay is performed with the Van Slyke manometric apparatus. In the developed technic, a 45-sec. reaction period is used, and a large liquid-gas interphase is provided with the purpose of preventing the oxygen diffusion from becoming a rate-limiting factor. Under the devised methodological conditions, the rate of oxygen evolution closely follows a parabolic curve which permits the calculation of the initial activity velocity of the enzyme. The oxygen evolvement is directly proportional to the quantity of catalase present in the analyzed sample. Less than 4 min. are required for each catalase measurement.**

SEVERAL GASOMETRIC METHODS have been used for determination of catalase activity by direct measurement of the rate of oxygen evolvement resulting from the enzymic decomposition of hydrogen peroxide, but during the last 40 years, no procedure has been described in which the Van Slyke manometric apparatus is employed for the assay of this enzyme. Because of the explosive character of the catalase reaction and the fast inactivation of the enzyme by the hydrogen peroxide substrate, a rapid method is required for reliable estimation of the initial velocity rate.

A suitable fast method for gasometric catalase determination has recently been devised by Greenfield and Price (*4*); in that procedure, a special apparatus is employed in which the evolved oxygen is measured by a pressure transducer and a continuous recorder system. In view of the simplicity and general availability of the Van Slyke mano-

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metric apparatus, it was considered advisable to develop a new method for catalase assay with the use of that equipment.

In the technic described in the present paper special consideration has been given to the basic features of the catalase assay which have proved advantageous in the Greenfield and Price method. The released oxygen is measured manometrically after a very short reaction time and conditions have been selected and arranged which provide a large surface area for the assay sample in the Van Slyke extraction chamber, thus facilitating the diffusion of oxygen across the gas-liquid interphase during the reaction period.

It was empirically observed by the author that the rate of oxygen evolvement under the conditions of the developed manometric procedure very closely followed a parabolic curve and was directly proportional to the amount of purified catalase and quantity of blood and tissue homogenate present in the sample. Since the findings were consistent and the results were highly reproducible, these observations permitted the development of a fast and reliable method for determination of the catalase activity in blood and tissue samples.

## Method

A conventional Van Slyke manometric apparatus with a gas extraction chamber calibrated at 0.5, 2.0, 10.0, and 50.0 ml. is used in the catalase assay procedure. The general technic for handling the apparatus must be employed in carrying out the enzyme activity test. It should be pointed out that for special reasons which will be explained later in this paper the catalase test is performed with 3.0 ml. of solution (S) and that the pressure readings are made at a volume (a) of 7.0 ml. (Fig. 1).

### Reagents

*Hydrogen peroxide solution, 1.0 M* Prepare by adding 2.9 ml. of 30% $H_2O_2$ solution to 22.1 ml. of 0.01 M phosphate buffer, pH 6.9. The pH of the commercial hydrogen peroxide stock solution should be checked and its molarity verified by permanganate titration or by measurement of the optical density of diluted samples at 240 m$\mu$.

*Phosphate buffer solution, 0.01 M pH 6.9* Prepare according to description made by Peters and Van Slyke (*8*).

*Caprylic alcohol*

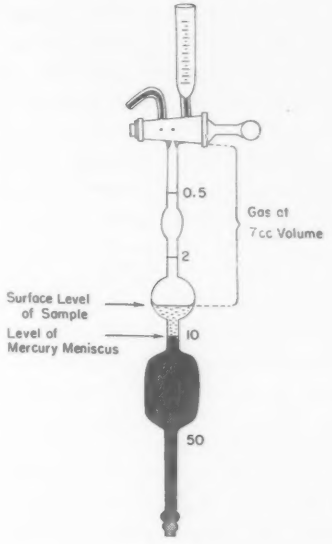


**Fig. 1.** Extraction chamber of Van Slyke manometric apparatus for use in gasometric catalase method. Gas is shown at 7.0-ml. volume for pressure reading.

## Procedure

### Transfer of Sample to Extraction Chamber

At the beginning of the test when the wash water is drawn off, the chamber fills with mercury. The chamber stop cock is closed, and the cock to the leveling bulb is left open. With a pipet, 0.9 ml. of phosphate buffer is measured into the cup of the extraction chamber.

The sample to be analyzed (hemolysate of 1:100 aqueous dilution, or tissue homogenate of suitable concentration) is drawn into a 100-$\mu$l. Linderstrøm-Lang constriction pipet and is added to the phosphate buffer in the cup. During the addition, the pipet is held in a vertical position with its point placed under the surface of the buffer. The transfer of the sample from the pipet is made slowly, the last part being ejected with the use of a rubber bulb. To insure quantitative transfer, the Linderstrøm-Lang pipet is rinsed by drawing phosphate buffer from the cup to the 100-$\mu$l. constriction mark with subsequent delivery of the pipet content to the cup sample. The 1.0-ml. volume in the cup is then introduced into the chamber without admitting any air.

Another 0.9 ml. of phosphate buffer is measured into the cup and similarly run into the chamber. This second portion of phosphate

buffer serves to wash into the chamber residuals of the sample which may adhere to the bottom of the cup. With a medicine dropper, four drops of caprylic alcohol are then placed in the cup, and a sufficient part of this reagent is admitted to the chamber to bring the mercury meniscus to the 2.0-ml. mark. A few drops of mercury are then added to the small amount of caprylic alcohol remaining in the cup, and with the chamber stop cock closed, the sample is ready for receipt of the hydrogen peroxide reagent.

#### Addition of Hydrogen Peroxide Reagent

The addition of the hydrogen peroxide reagent to the sample in the extraction chamber is made with a 1.0-ml. Ostwald-Van Slyke pipet equipped with stop cock and a rubber tip. Between use, this pipet is conveniently kept standing in a tall, narrow beaker placed immediately to the right of the Van Slyke apparatus.

The 1.0M $H_2O_2$ reagent is drawn into the pipet which is then inserted into the cup with the rubber tip pressed against the bottom. The pipet cock is opened, and if held in correct position, there will be no flow from the pipet. The admission of the 1.0 ml. of hydrogen peroxide solution to the chamber is made by means of the chamber stop cock. Immediately before the opening of that stop cock, a stop watch is started; if an electrical stop watch operated by a foot switch is employed, the starting of the watch can be made at the same second the chamber stop cock is opened. When the 1.0 ml. of hydrogen peroxide reagent has been transferred through the mercury seal into the extraction chamber, the chamber stop cock is closed, and the Ostwald-Van Slyke pipet is withdrawn and replaced in the beaker for storage. The bore of the chamber stop cock is then sealed with the mercury remaining in the cup. Between 9 and 11 sec. are required for the delivery of the hydrogen peroxide reagent to the chamber.

#### Reaction Period

By means of the leveling bulb, the mercury in the chamber is lowered smoothly until the mercury has fallen to the 10.0-ml. mark. The stop cock leading to the leveling bulb is closed and is subsequently used for adjustment of the mercury meniscus exactly to the 10.0-ml. mark (Fig. 1). This adjustment must have been accomplished by the time a 20-sec. reading is registered on the stop watch. The initial pressure ($p_o$) exerted by the sample is quickly read on the manometer and the shaking of the chamber is immediately started. The shaking is continued

for 45 sec. at a rate of 250 rpm (the shaking is started when the stop watch shows a 20-sec. reading and is stopped at 65 sec.).

During the period of shaking, the cock is occasionally opened with a smooth gradual motion to admit mercury from the leveling bulb to keep the mercury meniscus close to the 10.0-ml. mark. The retainment of the mercury meniscus in this narrow part of the extraction chamber assures a small area of contact between the hydrogen peroxide-containing sample and the mercury, and provides a large surface area between the sample and the gas phase in the chamber.

When the 45-sec. shaking period has been reached (when the watch shows the total time of 65 sec.), the shaking of the chamber is stopped, and the mercury meniscus is exactly adjusted to the 10.0-ml. mark (Fig. 1). The pressure reading ($p_1$) is immediately made on the manometer and the temperature is recorded.

The stop cock leading to the leveling bulb is opened and the sample is removed from the chamber by suction. The cup and the extraction chamber are then washed twice with 10–15 ml. of distilled water.

A complete catalase test including the measuring of the sample and reagents into the pipets and the washing of the chamber can be made in less than 4 min.

### Blank Value

A blank value ($c$) is determined by performing a test with 1.9 ml. of phosphate buffer, 4 drops of caprylic alcohol, and 1.0 ml. of 1.0 M $H_2O_2$ reagent. The blank value recorded for the 45-sec. test is usually about 6 mm. The mean time required for a blank determination is 3¼ min.

### Calculation of Catalase Activity

The pressure of oxygen evolved in the 45-sec. reaction period is $P = p_1 - p_o - c$, where $c$ is the pressure exerted by the blank.

The $P$ value is converted to microliters of oxygen (at 0° and 760 mm. Hg) by multiplication with the appropriate factor listed in Table 1. Since the catalase reaction rate as recorded in the present manometric procedure strictly follows a parabolic curve, the initial velocity constant ($k$) is calculated on the basis of the following formula:

$$k = \frac{q}{\sqrt{t}}$$

where $q$ = quantity of oxygen evolved and $t$ = time in seconds.

The initial reaction values may be expressed as microliters of oxy-

**Table 1.** FACTORS FOR CALCULATION OF AMOUNT ($\mu$L.) OF OXYGEN EVOLVED

| *Temp.°* | *Factor* |
|---|---|
| 15 | 8.845 |
| 16 | 8.811 |
| 17 | 8.777 |
| 18 | 8.743 |
| 19 | 8.710 |
| 20 | 8.677 |
| 21 | 8.644 |
| 22 | 8.611 |
| 23 | 8.579 |
| 24 | 8.547 |
| 25 | 8.516 |
| 26 | 8.484 |
| 27 | 8.453 |
| 28 | 8.423 |
| 29 | 8.392 |
| 30 | 8.360 |
| 31 | 8.331 |
| 32 | 8.302 |
| 33 | 8.271 |
| 34 | 8.242 |
| 35 | 8.213 |

Factors were calculated for conditions of $S = 3.0$ ml. and $a = 7.0$ ml.

gen evolved per second (Equation 1), micromoles of oxygen evolved per second (Equation 2), or micromoles of hydrogen peroxide decomposed per second (Equation 3):

$$k = \frac{P \times f}{\sqrt{t}} \quad (1)$$

$$k = \frac{P \times f}{22.4\sqrt{t}} \quad (2)$$

$$k = \frac{P \times f \times 2}{22.4\sqrt{t}} \quad (3)$$

where $P$ = observed pressure of oxygen evolved, expressed in millimeters mercury and $f$ = factor listed in Table 1.

The enzymic velocity values calculated from the above equations should subsequently be corrected for the temperature at which the test was conducted to convert activities to those exhibited by catalase at 25°. This correction is made by use of the factors recorded in Table 2.

As an example, a diluted human blood sample equivalent to 1 $\mu$l. of whole undilutel blood after 45 sec. of shaking at a temperature of 29° produced an 88.5 mm. increase in pressure (corrected for blank value).

**Table 2.** Factors for Conversion of Blood Catalase Activities Observed at Various Temperatures to Those Exhibited at 25°

| *Temp.°* | *Factor* |
|---|---|
| 15 | 1.1374 |
| 16 | 1.1292 |
| 17 | 1.1200 |
| 18 | 1.1096 |
| 19 | 1.0980 |
| 20 | 1.0839 |
| 21 | 1.0711 |
| 22 | 1.0542 |
| 23 | 1.0389 |
| 24 | 1.0198 |
| 25 | 1.0000 |
| 26 | 0.9773 |
| 27 | 0.9558 |
| 28 | 0.9301 |
| 29 | 0.9035 |
| 30 | 0.8758 |
| 31 | 0.8513 |
| 32 | 0.8231 |
| 33 | 0.7971 |
| 34 | 0.7680 |
| 35 | 0.7404 |

According to Equation 1, this corresponds to:

$$\frac{88.5 \times 8.392}{\sqrt{45}} = \frac{742.7}{6.708} = 110.7\ \mu\text{l. } O_2\text{/sec. in initial reaction}$$

The values, expressed as micromoles of oxygen evolved per second (Equation 2) and micromoles of hydrogen peroxide decomposed per second (Equation 3) are, respectively, 4.94 and 9.88. A conversion of these figures to enzymic activity at 25° (Table 2) yields values of 92.9 $\mu$l. of oxygen evolved per microliter undiluted blood per second; 4.15 $\mu$M of oxygen evolved per microliter undiluted blood per second; and 8.30 $\mu$M of hydrogen peroxide decomposed per microliter undiluted blood per second.

If desired, the shaking reaction period may be changed from 45 sec. ($\sqrt{45} = 6.708$) to 30 sec. ($\sqrt{30} = 5.477$) or 60 sec. ($\sqrt{60} = 7.746$).

### Cleaning the Chamber with Chromic-Sulfuric Acid Solution

When the manometric apparatus is used for catalase activity determinations, it is occasionally necessary to clean the extraction chamber

with a chromic-sulfuric acid solution as described by Peters and Van Slyke (*8*); because of the importance of the pH of the reaction mixture in the catalase determination, the acidity of the final wash water should be checked after this cleaning. After removal of the chromic–sulfuric acid solution, a satisfactory pH (between 6.5 and 7.0) was achieved when the chamber was washed successively with three portions of water, one portion of 0.5 N NaOH, and three additional portions of water, the volume of each washing solution being between 25 and 50 ml.

## Experimental

### Catalase Reaction Rate

For determination of the correlation between time of reaction and quantity of oxygen evolved, experiments were conducted with samples of human hemolysates and liver homogenates, and with commercial catalase preparations. The oxygen evolution was recorded after various times of shaking of the chamber; the reaction periods chosen were 15, 30, 45, 60, 90, and 120 sec. of shaking. Since it is not possible to make accurate readings on the manometer during the shaking of the extraction chamber, the tests were made successively on equal aliquots of the same catalase-containing samples. Duplicate analyses were performed in all the studies and usually showed very close agreement.

The constructed graphs regularly displayed a shape indicating a parabolic curve. In order to prove the parabolic reaction rate, the quantities of oxygen evolved were plotted against $\sqrt{t}$. In all the experiments this plotting resulted in a straight line passing through the origin (the origin representing the time at which the shaking was started). These findings verify the parabolic nature of the catalase reaction rate under the present conditions of assay (*3*). At the various times of reaction, a close correlation was found between the amount of enzyme present and the recorded activity values; as an example, the values observed for a blood sample are presented in Fig. 2. Similar proportionality was encountered in assays of freshly prepared solutions of purified catalase samples (Fig. 3).

### Relationship between Shaking Rate of Chamber and Catalase Reaction

A shaking rate of 250 rpm of the driving wheel was chosen for the manometric catalase procedure because in the determinations of blood and tissue samples, nearly identical activities were observed with 240, 250, and 260 shakes per minute. Slightly lower values were recorded at 210 rpm, and a markedly reduced rate of oxygen evolution was found

without shaking of the chamber (15% of that observed with 250 shakes per minute).

At the selected 250 rpm, approximately similar catalase activity values were noted for the 45-sec. shaking period when the shaking of

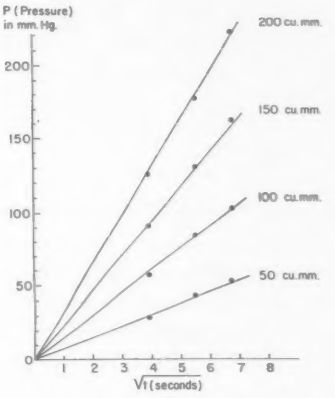


**Fig. 2.** Correlation between time of reaction (shaking period), volume of blood used, and quantity of oxygen evolved. The manometric readings are expressed in mm. Hg corrected for blank values. A 1:100 diluted human blood sample was used in test.

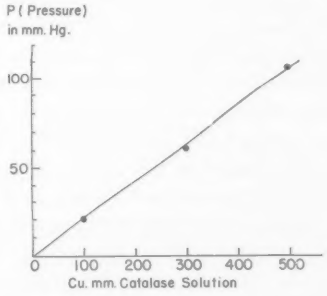


**Fig. 3.** Catalase activities exhibited by purified preparation recorded at various enzyme concentrations under standard conditions of manometric method (100 $\mu$l. of solution employed is equal to 5.7 $\mu$g. of catalase). Reaction time (shaking period), 45 sec.

the chamber was started either 20 or 25 sec. after the initiation of the hydrogen peroxide addition to the sample. The choice of a 45-sec. shaking period, from 25 to 70 sec. instead of the described period starting at 20 sec. and discontinued at 65 sec., may be preferable to analysts who are not routinely trained in the handling of the Van Slyke apparatus.

### Influence of Hydrogen Peroxide Concentration on Catalase Reaction Rate

The catalase activities of blood and tissue samples were found to be nearly independent of the $H_2O_2$ concentration within the range of 0.625

to 1.25 M (final concentrations 0.208 to 0.417 M). Somewhat lower catalase values were noted with the use of 0.3125 and 2.50 M $H_2O_2$ reagent solutions.

Under the technical conditions described in the present paper most of the hydrogen peroxide substrate remains undecomposed during the test even at the highest catalase reaction rates measurable by the manometric procedure. The evolution of oxygen exerting a 400-mm. Hg pressure is equivalent to a decomposition of 304 $\mu$M of hydrogen peroxide which represents only 30.4% of the 1000 $\mu$M of $H_2O_2$ (0.1 ml. of 1.0 M $H_2O_2$) added to the catalase-containing sample in the extraction chamber.

### Effect of Temperature on Catalase Activity Rate

A thorough investigation was made of the effect of temperature on human blood catalase activity as determined by the present manometric method. Five human blood samples were included in the study. The catalase activities of these samples were measured over the range of 15–35°. The control of the reaction temperature was accomplished through continuous circulation of water with a special constant temperature through the chamber jacket. Large needles were inserted through the upper and lower rubber stoppers of the jacket, and rubber tubing was attached to the heads of the needles. The circulating water was admitted to the jacket through the lower needle.

When a change in the temperature was made, the extraction chamber was left filled with mercury for 20 min. to assure a more uniform temperature level in the apparatus. After each test, the sample temperature was immediately measured.

The phosphate buffer solution employed in the tests was maintained in a water bath at the same temperature; hemolysate samples were kept in a refrigerater at 4° between assays. The experiments were started at 35°, and the temperature was then gradually lowered to 15°. In order to verify that no measurable degradation of the blood catalase had occurred during the long experimental period, the temperature was finally raised to the original level of 35°. Essentially similar values were recorded in the initial and final experiments conducted at the same temperature.

The effect of the temperature on the blood catalase activity of the five samples studied was found to be quite similar. Since the temperature curve is not linear, the observed graph is presented in Fig. 4 and the mean $Q_5$ and $Q_{10}$ values are listed in Table 3.

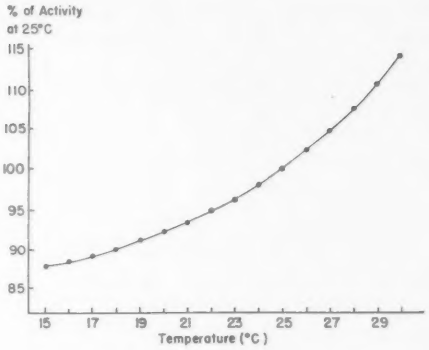


**Fig. 4.** Percentage values of oxygen evolved (expressed in ml. at 0°, 760 mm. Hg) by catalase in human blood samples at various temperatures, as compared with activities recorded at 25°.

**Table 3.** $Q_5$ AND $Q_{10}$ VALUES FOR BLOOD CATALASE REACTION AS DETERMINED BY MANOMETRIC PROCEDURE

| *Temp. range °* | $Q_5$ | *S.E.* | $Q_{10}$ | *S.E.* |
|---|---|---|---|---|
| 15–20 | 1.048 | 0.010 | | |
| 20–25 | 1.086 | 0.010 | | |
| 25–30 | 1.143 | 0.012 | | |
| 30–35 | 1.192 | 0.011 | | |
| 15–25 | | | 1.139 | 0.025 |
| 20–30 | | | 1.240 | 0.021 |
| 25–35 | | | 1.345 | 0.022 |

## Velocity Values Observed for Commercial Catalase Preparations

On the basis of the initial velocity rate determined with the manometric method, the turnover number for the enzyme in a purified catalase powder* prepared from beef liver was found to be 5,120,000. The quantity of enzyme present in the catalase solution used in the test was confirmed through measurement of the optical density of the stock solution at 280 m$\mu$ in a spectrophotometer† and by assay according to the procedure of Beers and Sizer (*1*); the observed enzyme content of the beef liver catalase powder agreed closely with that reported by the Sigma Chemical Co. (Lot C41B-085). An essentially similar turnover number of 5,180,000 was found for a catalase preparation obtained from Worthington Biochemical Corp., Freehold, N. J. (Lot 5553-78). The high turnover number for purified catalase recorded with the present manometric technic indicates the validity of that procedure.

*Sigma Chemical Company, St. Louis, Mo.
†Model DU, Beckman Instruments Inc., Palo Alto, Calif.

### Catalase Activities of Human Blood Specimens and Liver Tissue Homogenates

Catalase activity measurements were performed on 100 human blood samples obtained from patients at St. Louis Chronic Hospital (average age of subjects, 75 years). Hematocrit and hemoglobin determinations were made by conventional methods. Hemolysates of the specimens were prepared by adding 100 $\mu$l. of each blood sample to 9.9 ml. of redistilled water. The average initial velocity rate calculated from the manometric catalase assays was 115 $\mu$l. of oxygen evolved per microliter undiluted blood per second (S.D. of dist., 35); this activity rate is somewhat higher than those previously reported with gasometric technical procedures (*2*). The average difference between duplicate measurements of the samples was 2.6%. When expressed per microliters of red blood cells, the mean oxygen evolution was 286 $\mu$l./sec. (S.D. of dist., 89); the corresponding value per milligrams of hemoglobin was 842 $\mu$l. (S.D. of dist., 265).

For determination of the catalase activity of liver tissue, 10 specimens were obtained fresh at autopsy, and 1.0% aqueous homogenates were prepared with a Kontes Duall type tissue grinder. The mean catalatic value observed for human liver tissue was 93 $\mu$l. of oxygen evolved per milligram tissue per second. This activity rate is of the same order of magnitude as that reported by Greenstein *et al.* (*3*) for normal mouse liver.

## Discussion

The factors responsible for the oxygen evolution at a rate corresponding to a parabolic curve are not known, but the experimentally established consistency of this type of catalase reaction under the employed technical conditions permits an accurate calculation of the initial activity velocity. Attempts will not be made to correlate this empirical finding with the available general information concerning the aspects of catalase kinetics.

It is of interest to note that a calculation of gasometric catalase data reported by Morgulis (*7*) and Maisin and Pourbaix (*6*) indicates a tendency toward a parabolic curve; this was noted by plotting the published values of oxygen evolvement against the square root of reaction times. However, the graphs constructed on the basis of these previously reported catalase activity measurements do not pass directly through the zero point, possibly due to the fact that the technics used by those authors differ in many respects from the procedure described in this paper.

In the present method, the use of a 3.0 ml. sample volume and the maintenance of the mercury meniscus at the 10.0-ml. mark were chosen with the purpose of establishing a large liquid-gas interphase area (Fig. 1). The surface of the sample in the Van Slyke extraction chamber is approximately 1.3 sq. cm./ml. of sample volume; this is an even higher ratio than that of 0.5 sq. cm./ml. of solution calculated on the basis of the size and shape of the reaction flask used in the method by Greenfield and Price (*4*). The conditions are very satisfactory for swirling the sample over the inside of the glass surface in both these rapid gasometric technics.

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# A Procedure for Quantitative Determination of the Diaphorase Activity of Connective Tissue

*John Esben Kirk*

**A quantitative procedure is described in which the diaphorase activity of samples with a high connective-tissue content is measured visually through determination of the decolorization time of 2,6-dichlorophenolindophenol in the presence of DPNH and NaCN. The decolorization time values are directly converted to standard Mahler diaphorase units.**

DIAPHORASE is a flavin-containing enzyme which catalyzes the oxidation of DPNH (reduced diphosphopyridine nucleotide) in the presence of an electron acceptor. The procedure generally used for determination of the activity of this enzyme is based on spectrophotometric measurement of the reduction of 2,6-dichlorophenolindophenol at 600 mμ. As pointed out by Mahler (*4*), this technic is not applicable to tissue homogenates exhibiting high turbidity. A method was therefore developed for the assay of diaphorase in samples with a high connective-tissue content.

In that procedure described in the present paper, the decolorization time of 2,6-dichlorophenolindophenol is measured visually in the presence of DPNH and NaCN. The number of Mahler units present in the sample is read from a curve (Fig. 1). The curve was constructed on the basis of decolorization times recorded at 25° with the use of purified commercial diaphorase preparations.* The activities of these preparations were measured spectrophotometrically by Mahler's (*4*) method immediately before and after their use in the construction of the

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*Worthington Biochemical Corporation, Freehold, N. J. and Nutritional Biochemicals Corporation, Cleveland, Ohio.

standard decolorization time curve; the stability of the purified diaphorase samples was assured through addition of bovine albumin to the enzyme solutions. A very close agreement was observed between the decolorization time curves constructed on the basis of the tests con-

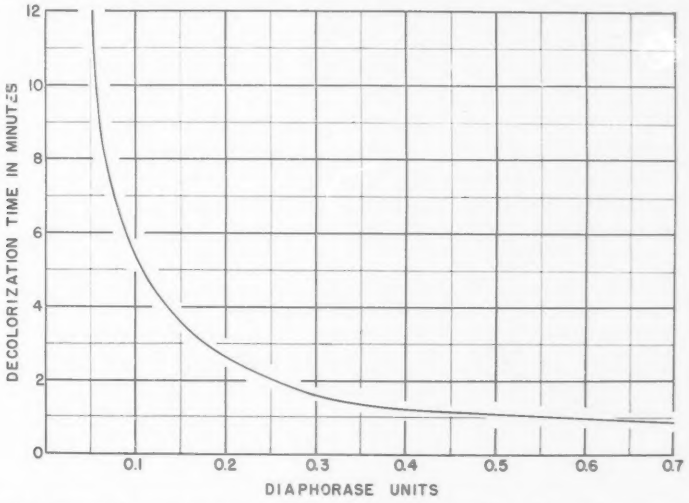


**Fig. 1.** Standard curve for measurement of diaphorase activities of tissue homogenates on basis of decolorization time tests at 25°

ducted with the two commercial diaphorase preparations. The recorded $Q_{10}$ for the decolorization reaction between 20° and 30° was 1.48.

## Method

### Reagents

1. *Tris buffer solution, 0.1 M, pH 7.5*
2. *DPNH solution, 6.0 mM*
3. *NaCN solution, 2.7 mM*
4. *2,6-dichlorophenolindophenol solution,* 0.06 mM* The molar extinction coefficient observed for the compound at 600 mμ is 19,100 (*1, 2*).

### Enzyme Test

The test is conducted at room temperature. After 0.5 ml. of Tris buffer, 0.1 ml. of DPNH solution, and 0.1 ml. of NaCN solution are

*Eastman Kodak Co., Rochester, N. Y.

pipetted into a 100 × 13-mm. test tube, a freshly prepared aqueous tissue homogenate sample containing 10–200 mg. of fresh tissue is then added, and the volume adjusted to 1.7 ml. with distilled water. The reaction is started by the addition of 1.0 ml. of the 2,6-dichlorophenolindophenol reagent. The decolorization time is recorded with a stop watch, and the temperature of the sample is measured. The number of Mahler diaphorase units present in the sample is read from the standard curve (Fig. 1) or from a table (Table 1). The unit value is subsequently corrected for the temperature at which the test was conducted by multiplication with the appropriate factor listed in Table 2.

The quantities of tissue used in the test should be chosen so as to give decolorization times corresponding to the sloping part of the curve. Under the conditions of the procedure, no decolorization was observed

**Table 1.** Diaphorase Units of Tissue Samples Determined on the Basis of Decolorization Time at 25°

| *Min.* | *Sec.* | *Units* | *Min.* | *Sec.* | *Units* | *Min.* | *Sec.* | *Units* | *Min.* | *Sec.* | *Units* |
|---|---|---|---|---|---|---|---|---|---|---|---|
| 2 | 0 | 0.254 | 3 | 0 | 0.178 | 4 | 0 | 0.135 | 5 | 0 | 0.108 |
| 2 | 5 | 0.246 | 3 | 5 | 0.173 | 4 | 5 | 0.132 | 5 | 5 | 0.106 |
| 2 | 10 | 0.238 | 3 | 10 | 0.169 | 4 | 10 | 0.130 | 5 | 10 | 0.104 |
| 2 | 15 | 0.230 | 3 | 15 | 0.166 | 4 | 15 | 0.127 | 5 | 15 | 0.103 |
| 2 | 20 | 0.224 | 3 | 20 | 0.161 | 4 | 20 | 0.125 | 5 | 20 | 0.101 |
| 2 | 25 | 0.217 | 3 | 25 | 0.157 | 4 | 25 | 0.123 | 5 | 25 | 0.099 |
| 2 | 30 | 0.211 | 3 | 30 | 0.154 | 4 | 30 | 0.120 | 5 | 30 | 0.097 |
| 2 | 35 | 0.205 | 3 | 35 | 0.150 | 4 | 35 | 0.117 | 5 | 35 | 0.096 |
| 2 | 40 | 0.199 | 3 | 40 | 0.147 | 4 | 40 | 0.115 | 5 | 40 | 0.095 |
| 2 | 45 | 0.194 | 3 | 45 | 0.143 | 4 | 45 | 0.114 | 5 | 45 | 0.093 |
| 2 | 50 | 0.188 | 3 | 50 | 0.141 | 4 | 50 | 0.111 | 5 | 50 | 0.092 |
| 2 | 55 | 0.183 | 3 | 55 | 0.138 | 4 | 55 | 0.110 | 5 | 55 | 0.091 |

**Table 2.** Factors for Conversion of Diaphorase Units to Standard Units at 25°

| *Temp.* | *Factor* |
|---|---|
| 20 | 1.235 |
| 21 | 1.18 |
| 22 | 1.13 |
| 23 | 1.09 |
| 24 | 1.05 |
| 25 | 1.00 |
| 26 | 0.965 |
| 27 | 0.93 |
| 28 | 0.89 |
| 29 | 0.865 |
| 30 | 0.835 |

in reagent blanks or in tissue controls (arterial tissue and cerebellar tentorium) without addition of DPNH, over a period of several hours.

## Experimental

With the use of the present procedure, assays have been made of the diaphorase activity of human vascular tissue (*3*) and cerebellar tentorium. Determinations performed with different quantities of tissue usually provided values (expressed as units per gram of tissue) which agreed within 5%. As an example, experiments conducted at 22° with 10, 25, and 50 mg. of an arterial sample showed decolorization times of 6 min. 28 sec., 2 min. 39 sec., and 1 min. 16 sec., respectively. These values correspond to 0.0937, 0.226, and 0.460 Mahler units (corrected for temperature) present in the assayed tissue portions, or 9.37, 9.04, and 9.20 Mahler units per gram of tissue. Activities exhibited by supernatants of centrifuged arterial homogenates were found to be much lower than the diaphorase values recorded for the total homogenates. This observation emphasizes the necessity for a special procedure applicable to homogenates with a high connective tissue content.

The cerebellar tentorium was found to possess a relatively low diaphorase activity (average value, 0.6 Mahler units per gram). In the assay of this type of connective tissue, 100- and 200-mg. samples were employed to obtain suitable decolorization times.

## Discussion

In the evaluation of tissue diaphorase activities, the fact should be considered that cytochrome c reductase seems to be capable of catalyzing the reduction of 2,6-dichlorophenolindophenol in the presence of DNPH (*4*). The exact relationship between these two flavin-containing enzymes has not been established, but recent studies (*5*) support the contention that the enzymes are distinct entities. For that reason, it may be advisable to make both diaphorase and cytochrome c reductase determinations on the same tissue samples (*3*).

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# BOOK REVIEWS

**Blood Vessels and Lymphatics.** DAVID I. ABRAMSON, ED. 812 pp., Academic Press, New York, N. Y., $26.

This is a unique compilation by an array of international authorities in their subspecialties of both currently accepted and debatable views of embryology, anatomy, pharmacology, and biochemistry of the vascular systems.

Dr. Abramson has overcome the hurdle of integrating and organizing, without altering basic viewpoints, this multifaceted subject. Each section is followed by an extensive, modern, international bibliography. References are readily available to viewpoints different from those presented by the specific author. The illustrations—diagrammatic, photographic, and electronmicroscopic—are ample and clear.

The various authors are to be complimented on the succinct manner in which the broad scope from basic physiological research to anatomic changes has been covered.

This text fills a void in the physiology, biology, chemistry and pathology laboratories for each of which it is wholeheartedly recommended for student and teacher alike.

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VICTOR TCHERTKOFF, M.D.

# the Clinical Chemist

## 16th National Meeting AACC
## Aug. 17-20, 1964, Boston, Mass.

Norbert Benotti, General Chairman of the 16th National Meeting, Boston, Mass., announced that all plans are being set to make the Boston Meeting the most successful thus far in the history of the AACC. To this end, he is appealing to every member of the Association to suggest some constructive ideas that may be formulated in the 1964 program. Any ideas relative to any aspect of the Meeting would be appreciated. Address all communications to NORBERT BENOTTI, *Boston Medical Laboratory, 19 Bay State Road, Boston 15, Mass.*

## Protides of the Biological Fluids

At the XIIth Annual Colloquium, "Protides of the Biological Fluids," to be held at Sint-Janshospitaal, Bruges, Belgium, Apr. 30–May 3, 1964, the following topics will be discussed: (1) Phylogeny of Proteins, (2) Tissue Proteins, (3) Urinary- Glyco- and Mucoproteins, and (4) Methods. Round-table conferences will be held in: (1) Tissue Proteins, (2) Methods, and (3) Proteins of Diagnostic Significance.

For application and communications, write to DR. HUB. PEETERS, Laboratory, St. Janshospital, Bruges, Belgium.

## Section News

### Michigan Section

The officers elected for 1963-64 are as follows: Daniel H. Basinski, Chairman; Emanuel Epstein, Chairman-Elect; Wendell T. Caraway, Secretary-Treasurer; Ruth D. McNair, Program Chairman; Victor Schelling, Professional Relations Chairman; Robert B. Foy, Standards Chairman; Roderick P. MacDonald, National Executive Committeeman.

### New York Metropolitan Section

The officers elected for 1963-64 are as follows: Abraham Saifer, Chairman; Walton Marsh, Chairman-Elect; Arthur Levy, Corresponding Secretary; Gerald Kessler, Secretary-Treasurer; Max M. Friedman, National Executive Committeeman.

The first meeting of the current season was held on Wednesday, October 23, at the New York Academy of Sciences. Dr. Howard J. Curtis, Chairman, Department of Biology, Brookhaven National Laboratories, Upton, New York, spoke on "The Cellular Basis of Aging."

A business meeting followed the scientific session, at which time reports were made concerning the proposed New York State Laboratory statutes and the new New York City Health Code which will go into operation

shortly. The Nominating Committee presented its slate of candidates for 1964-65.

### Northeast Section

The officers elected for 1963-64 are as follows: John Di Giorgio, Chairman; Louis Williams, Chairman-Elect; Joanne Boddie, Treasurer; Helen Lichwell, Secretary; Edward Roseland, Robert Champagne, and Estelle Koven, Nominating Committee.

Seven meetings were held in the 1962-63 season. In October, Dr. J. Vitale of the Harvard School of Public Health, spoke on clinical and experimental nutrition studies in Cali, Colombia, South America. A joint meeting was held in November with the Connecticut Section. Dr. Alfred Zettner of Yale-New Haven Medical Center discussed atomic absorption spectroscopy and its application to clinical chemistry. At the January meeting members heard Dr. H. Swachman of Harvard Medical School and the Children's Hospital Medical Center describe laboratory tests for the diagnosis of cystic fibrosis. In February Dr. J. C. Milby of the Boston University School of Medicine and Massachusetts Memorial Hospital discussed the laboratory diagnosis of adrenal-cortical disorders. A joint meeting was held in March with the Analytical Chemistry group of the American Chemical Society, with the speaker for the meeting Dr. Kei-Ischiro Fuma of the Harvard Medical School and Peter Bent Brigham Hospital. He told of the recent developments in emission and absorption flame photometry. In April, Dr. Bennie Zak of Wayne State University College of Medicine discussed the applications of agar-gel paper electrophoresis. At the final meeting in May, members heard Dr. R. B. McGandy of the Department of Nutrition of Harvard School of Public Health speak on calories, fats, and life-span.

### Ohio Valley Section

The officers elected for 1963-64 are as follows: Paige K. Besch, Chairman; Carl C. Smith, Chairman-Elect; James P. F. Murphy, Secretary; Bernard J. Katchman, National Executive Committeeman.

The October meeting was held at the Occupational Health Research and Training Facility, Cincinnati; the subject was Toxicology. The February meeting (a workshop on Fluorometry) was held at Miami Valley Hospital, Dayton. In June, a symposium on clinical microchemistry was held at Children's Hospital, Columbus. Participants on the program were R. P. MacDonald, W. R. Faulkner, and S. Meites. A panel discussion moderated by P. K. Besch followed.

### Pittsburgh Section

The officers elected for 1963-64 are as follows: Eugene W. Rice, Chairman; Nicholas G. Barbella, Secretary; Charles Lupu, Treasurer.

### Puget Sound Section

The officers elected for 1963-64 are as follows: Elizabeth K. Smith, Chairman; Richard A. Neve, Vice-Chairman; Marian C. Hunner, Secretary-Treasurer; Alex Kaplan, National Executive Committeeman; Edward J. Barron, Membership Chairman.

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Volume 9 Number 6 December 1963

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# CLINICAL CHEMISTRY

*Journal of the American Association of Clinical Chemists*

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**developments thick and heavy on the phosphate front**...No less than three new methods for automating phosphate determinations were reported at the last meeting of the American Association of Clinical Chemists—each accurate, each usable with the AutoAnalyzer, each possessing particular virtue for a particular circumstance. In addition a paper on Alkaline Phosphatase described the measurement of phosphate, instead of phenol, with readout in Bodansky units rather than King Armstrong units. We will be glad to supply reprints of all *as soon as available*.

**automated fluorometry for toxicity studies**... Of interest to those engaged in toxicity studies (e.g. aspirin poisoning) is a new micro method for salicylate determination (requires only *0.1 ml of fingertip blood*). Salicylate, dialyzed into a saline recipient stream, is mixed with alkali: the salicylates present, now fully converted to salicylic acid, are mixed with ethanol, and the resulting fluorescence is then measured. The micro method will measure down to 3 mg% salicylate.

**is there a librarian in the house?**... One of our prime responsibilities to our customers, no less than ourselves, we have always felt—is to keep abreast of developments on the analysis-automation front. Every time we sit down to update our AutoAnalyzer bibliography, we are awed at the swelling number of papers and references we come upon—and from every quarter of the globe, too, mind you.

Our last compilation, only six months old, is already hopelessly out of date (as you may know, if you have a copy).

In the interest of making this reference truly definitive and of maximum benefit for AutoAnalyzer users, we would vastly appreciate receiving (1) information on such omissions or errors as you may know of and (2) suggestions for improving its usefulness to you. If you lack a copy of the "current" listing, we'll be glad to send you one to check. An updated copy will eventually follow.

**blood alcohol—challenge worthy of anyone's mettle**... Intrigued by the problem of automating blood alcohol determinations, we have recently gone to great lengths to track down published papers on the subject and compile a summary of work done. We'll be glad to share it with anyone interested in bringing this stubborn holdout to heel.

Plenty of applause in store for he who solves the problem: the world is waiting for a *truly dependable* automated blood alcohol method. Kudos, anyone?

**two new "combos" succumb to simultaneous analysis**... No question about it: the increasing possibilities of *simultaneous* analysis of two or more physiological constituents has captured the imagination of pathologists everywhere. Those having AutoAnalyzer equipment adapted to simultaneous analyses (their increasing number is ample evidence of the burgeoning interest) can now add two new "combos" to their capability roster (1) creatinine/uric acid (2) and inorganic phosphate/uric acid. Both are AutoAnalyzer "N" methods and can be successfully run at 40 tests per hour.

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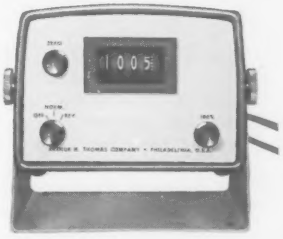


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*Journal of the American Association of Clinical Chemists*

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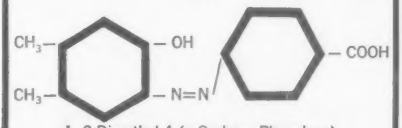
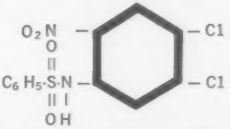



1, 2 Dimethyl 4 (p Carboxy Phenylazo) 5 Hydroxy Benzene

DCBN




1,2 Dichloro 4 Benzene Sulfonamido 5 Nitro Benzene

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(1) D. W. Woolley and J. M. Stewart, Biochem. Pharm. 11, 1163, (1962). (2) D. W. Woolley, Proc. Nat. Acad. Sci. Wash. 39, 6, (1953). (3) D. W. Woolley, Ibid, 41, 111, (1955). (4) D. W. Woolley, Cancer Res. 13, 327, (1953). (5) D. W. Woolley and G. Schaffner, Ibid, 14, 802, (1954).

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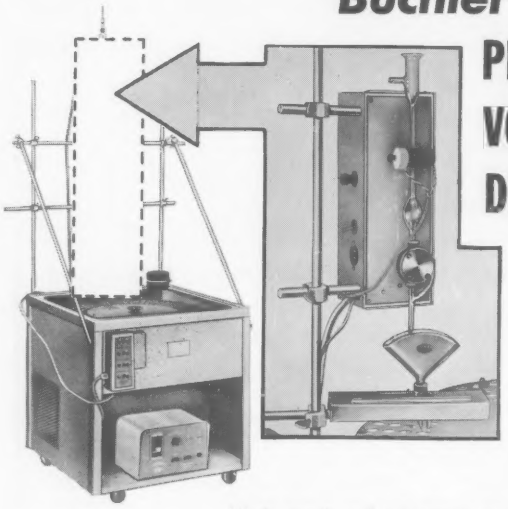

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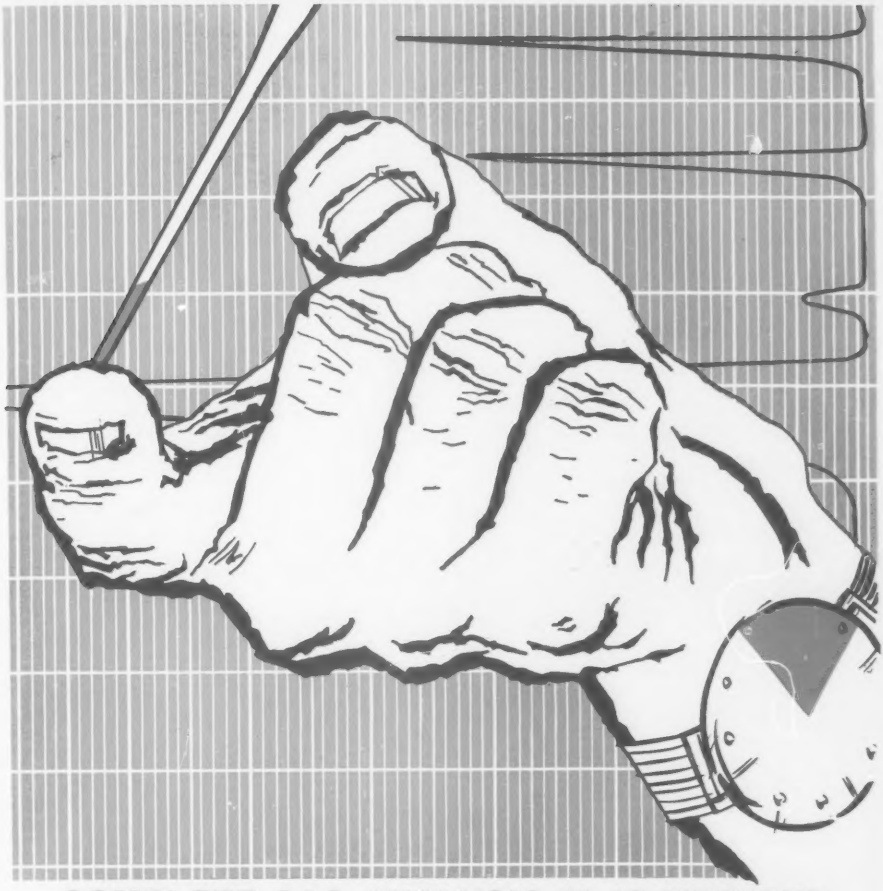

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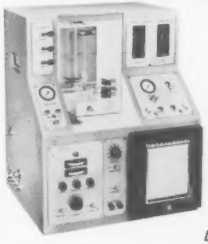

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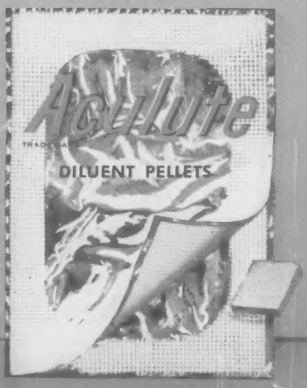


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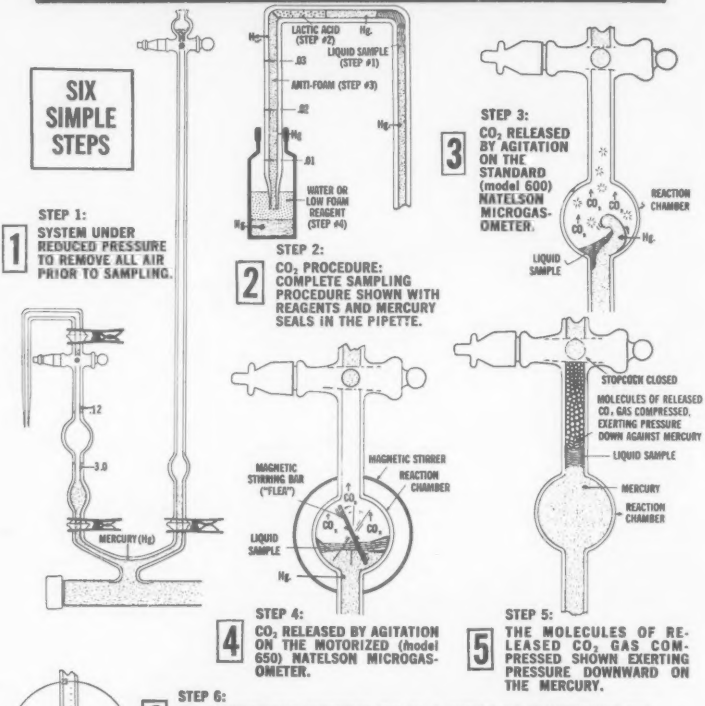
SIX SIMPLE STEPS
1
STEP 1:
SYSTEM UNDER REDUCED PRESSURE TO REMOVE ALL AIR PRIOR TO SAMPLING.
1.2
3.0
MERCURY (Hg)
LACTIC ACID (STEP #2)
Hg.
LIQUID SAMPLE (STEP #1)
.03
ANTI-FOAM (STEP #3)
.02
.01
WATER OR LOW FOAM REAGENT (STEP #4)
2
STEP 2:
$CO_2$ PROCEDURE: COMPLETE SAMPLING PROCEDURE SHOWN WITH REAGENTS AND MERCURY SEALS IN THE PIPETTE.
3
STEP 3:
$CO_2$ RELEASED BY AGITATION ON THE STANDARD (model 600) NATELSON MICROGAS-OMETER.
REACTION CHAMBER
LIQUID SAMPLE
4
STEP 4:
$CO_2$ RELEASED BY AGITATION ON THE MOTORIZED (model 650) NATELSON MICROGAS-OMETER.
MAGNETIC STIRRING BAR ("FLEA")
MAGNETIC STIRRER
REACTION CHAMBER
5
STEP 5:
THE MOLECULES OF RE-LEASED $CO_2$ GAS COM-PRESSED SHOWN EXERTING PRESSURE DOWNWARD ON THE MERCURY.
STOPCOCK CLOSED
MOLECULES OF RELEASED $CO_2$ GAS COMPRESSED, EXERTING PRESSURE DOWN AGAINST MERCURY
LIQUID SAMPLE
MERCURY
REACTION CHAMBER
6
STEP 6:
THE MANOMETRIC PRESSURE ($P_1$) AS A RESULT OF COMPRESSING THE LIBERATED $CO_2$ IN THE REACTION CHAMBER. THROUGH SELECTIVE ABSORPTION WITH 3N NaOH, $P_2$ IS OBTAINED.

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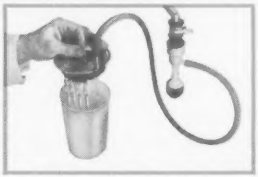

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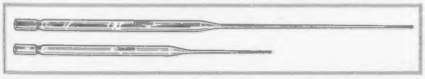

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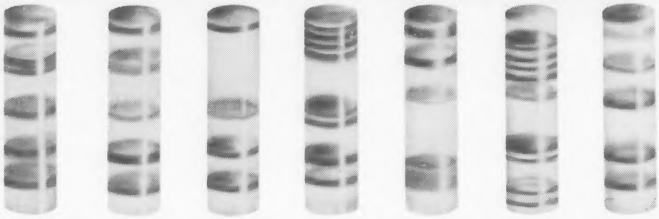

LDH in human liver | LDH in human serum (liver metastasis) | LDH in human serum (normal) | LDH in rat kidney | LDH in mouse epididymis | Esterase in human liver | Phosphorylase in algae

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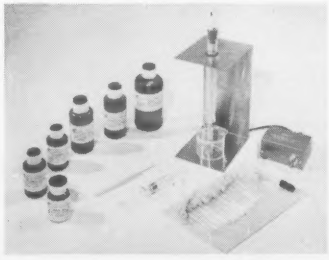

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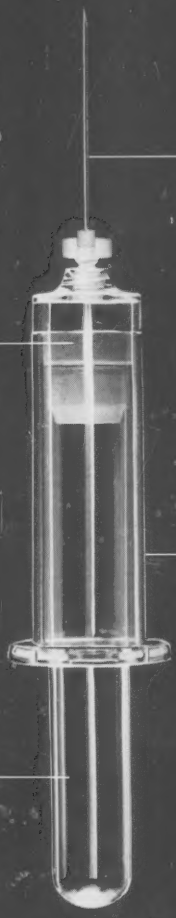

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REF: 1. Tarlov, et al., "Primaquine Sensitivity," Arch. Int. Med., *109*, 209, (1962).

2. Marks, et al., "Erythrocytic Glucose-6-Phosphate Dehydrogenase of Normal and Mutant Subjects," J.B.C., *236*, No. 1, (1961).

3. Kellermeyer, et al., "Hemolytic Effects of Therapeutic Drugs," J. Am. Med. Assoc., *180*, 388, (1962).

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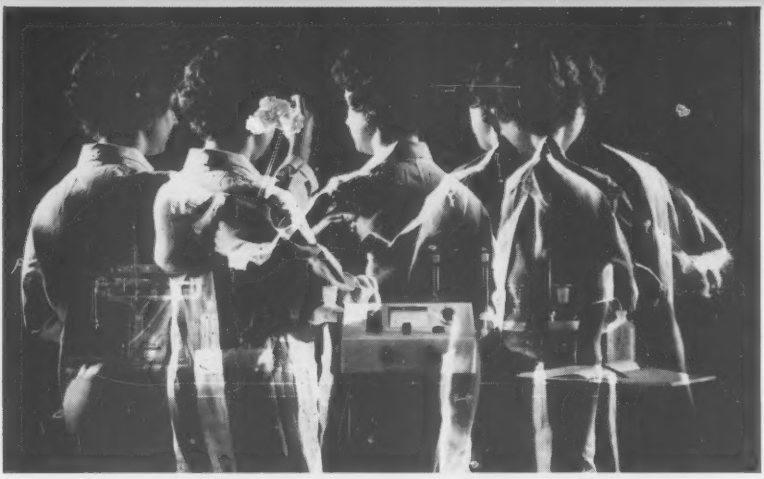

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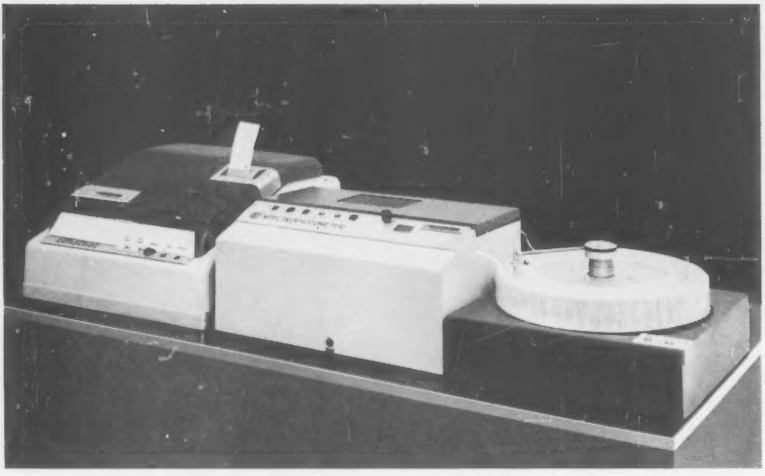

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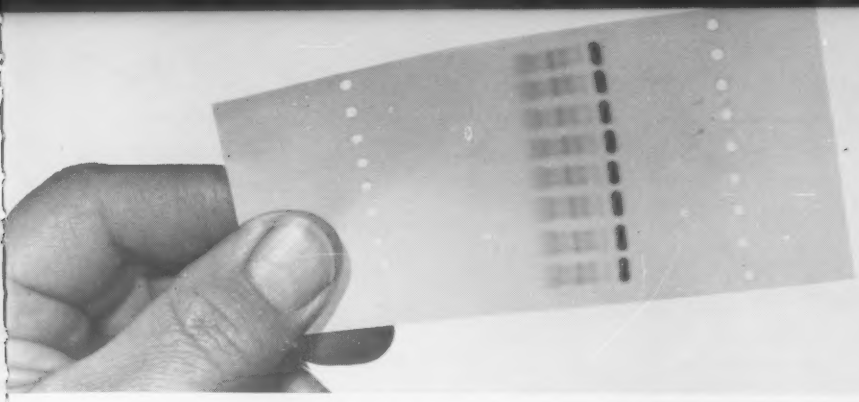

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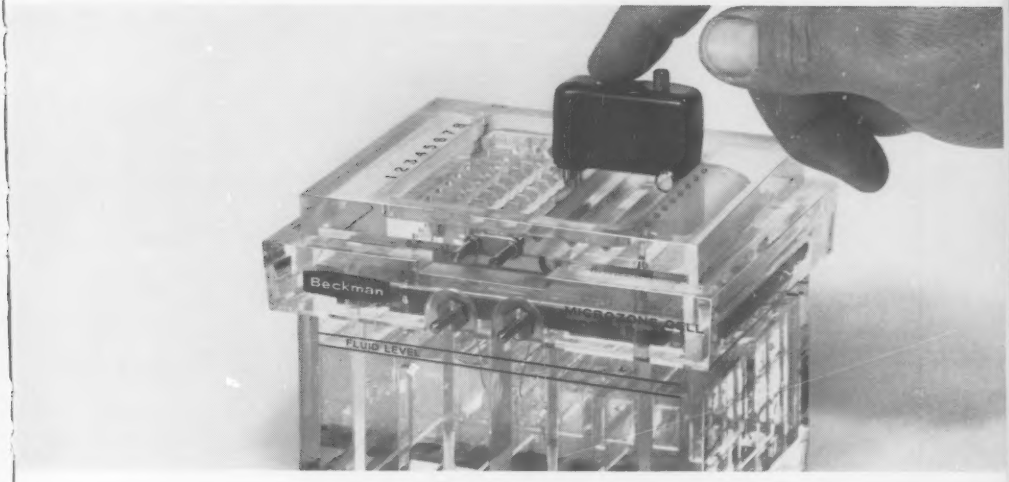


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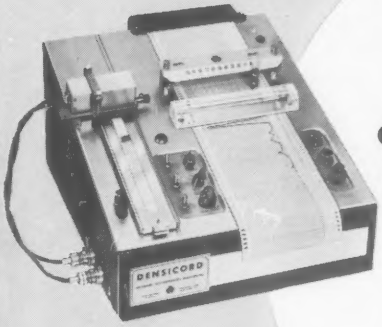


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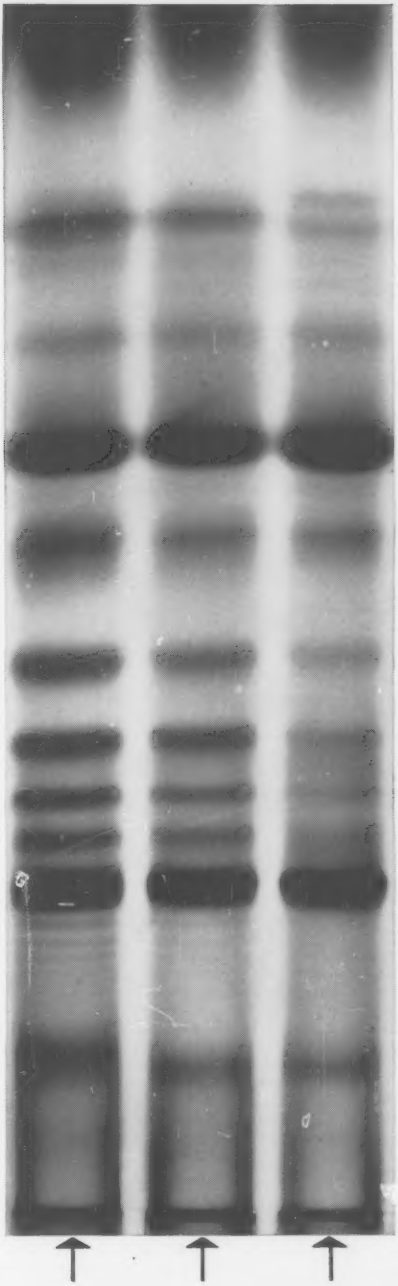

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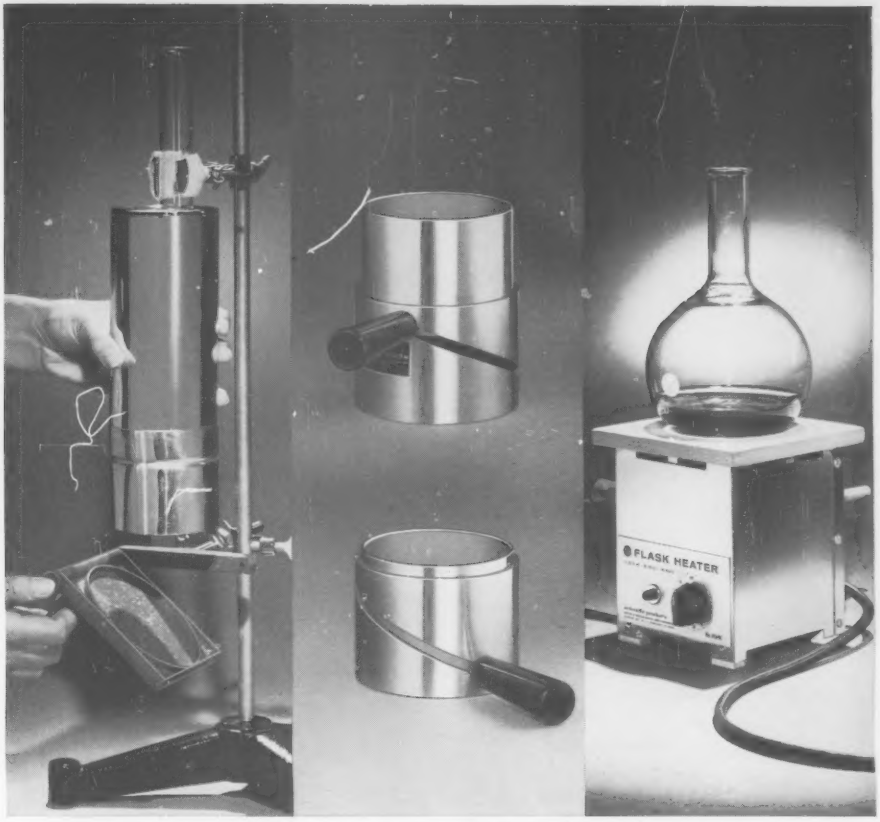


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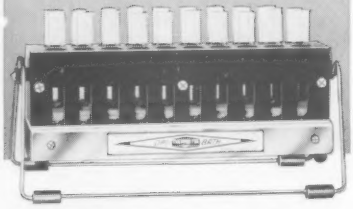

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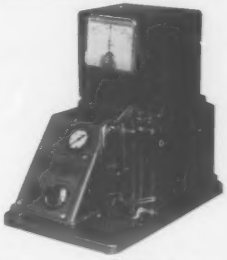

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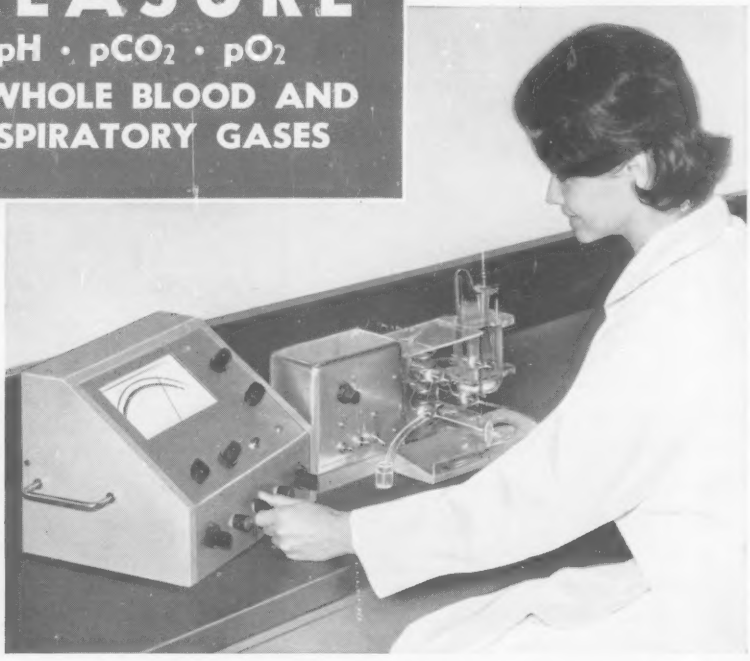

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